REVERSE GYRASE FROM ARCHAEBACTERIA

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by

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ABSTRACT

Reverse gyrase, an enzyme that positively supercoils DNA, was examined with regard to its distribution in thermophilic and mesophilic bacteria. It was discovered that, among the bacterial species tested, reverse gyrase activity was only present in the sulphur metabolizing archaebacteria. While screening for reverse gyrase, endonuclease and relaxing topoisomerase activities were also detected in some bacterial species.

Two archaebacteria, *Sulfolobus solfataricus* and the *Thermococcus*-like isolate AN1, were chosen as representatives of the major branches of archaebacteria which exhibit the greatest phylogenetic diversity among bacteria known to contain reverse gyrase. Reverse gyrase from these organisms was further investigated following the purification and characterization of the enzyme. The results suggested that reverse gyrase was not significantly different between *S. solfataricus* and AN1 in physical properties or in the catalytic conditions under which activity is maximal. Furthermore this similarity extends to the reverse gyrase from *Sulfolobus acidocaldarius* (Forterre *et al.*, 1985; Nadal *et al.*, 1988; Nakasu and Kikuchi, 1985) and *Desulfurococcus amylolyticus* (Slesarev, 1988).

The role of reverse gyrase in sulphur metabolizing archaebacteria was examined by attempting to elucidate the supercoiled state of their genomic DNA. The technique used for these experiments followed that of Worcel and Burgi, 1972 who extracted nucleoids from *E. coli* and ran them on sucrose gradients under different concentrations of ethidium bromide. The procedures necessary to succeed in using this technique were successfully developed using *E. coli* which was confirmed as containing a negatively supercoiled genome. However attempts to isolate nucleoids from *S. solfataricus* and AN1 were not successful and the supercoiled state of chromosomal DNA in these cells could not be determined.

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CORRECTIONS AND ADDITIONS

Page	Line	
8	8	'... the intrastrand twist number ...' should be '... the interstrand twist number.
19	8	The sentence starting 'Forterre *et al.*, 1989 ...' should include a comment that the inhibition of archaebacterial growth by coumarins does not constitute unequivocal evidence for the presence of DNA gyrase.
20	2	The sentence starting 'However it is different ...' should include a comment that topoisomerases coded for by the mitochondrial genome could possibly be different from those coded for by the eukaryotic nucleus DNA considering the likely eubacterial origin of the mitochondria.
21	12	'... covalent and non-covalent interactions with the 3' side and non-covalent interactions with the 5' side.' should be '... covalent and non-covalent interactions with the 5' side and non-covalent interactions with the 3' side.'.
33	7	'... large number of hydrophilic residues ...' should be '... large number of hydrophobic residues ...'.
37	18	The sentence starting 'Considering that the enzymatic ...' should include a comment to the effect that, it is unknown whether the eubacterial type I topoisomerase was derived from reverse gyrase or *vice versa*.
37	23	Section 1.3.4 'The distribution of reverse gyrase' should include the results of a recently published paper* which describes reverse gyrase activity in *Methanothermus fervidus, Methanopyrus kandleri* and *Archaeoglobus fulgidus*. The conclusion drawn is that the presence of reverse gyrase activity is linked to high-temperature growth rather than being the sole property of the sulphur metabolizing archaebacteria. * Bouthier de la Tour, C., Portemer, C., Nadal, M., Stetter, K.O., Forterre, P. and Duguet, M. (1990). Reverse gyrase, a hallmark of the hyperthermophilic archaebacteria. Journal of Bacteriology 172: 6803-6808.
45	25	'... is known to effect *in vivo* supercoiling ...' should be '... is known to affect *in vivo* supercoiling ...'.

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LIST OF ABBREVIATIONS

AN1 ..... *Thermococcus*-like organism from TRUCC
ATCC..... American Type Culture Collection
cccDNA..... Covalently closed circular DNA
Da..... Daltons
dsDNA..... Double-stranded DNA
DSM ..... German Collection of Microorganisms (Deutsche Sammlung von Mikroorganismen)
DTT ..... Dithiothreitol
EDTA..... Ethylenediaminetetraacetic acid
EPPS..... N-(2-Hydroxyethyl)piperazine-N'-3-propanesulfonic acid
form I DNA..... Negatively supercoiled plasmid DNA
form II DNA..... Nicked open circular plasmid DNA
form III DNA..... Linear plasmid DNA
FPLC..... Fast protein liquid chromatography
HPLC..... High performance liquid chromatography
hr..... Hour
IEF..... Isoelectric focusing
kDa..... Kilo Daltons
LB..... Luria-Bertani
Lk ..... Linking number
Lk° ..... Linking number of DNA free of torsional strain
MES ..... 2-(N-morpholino)ethanesulfonic acid
min ..... Minute
MOPS..... 3-(N-morpholino)propanesulfonic acid
OD..... Optical density
O/N..... Overnight
PAGE ..... Polyacrylamide gel electrophoresis
PMSF ..... Phenylmethanesulphonyl fluoride
rpm..... Revolutions per minute
RT..... Room temperature
SDS..... Sodium dodecyl sulfate
S layer..... Surface layer
sp ..... Species
ssDNA..... Single-stranded DNA
TEMED..... N,N,N',N'-tetramethylethylenediamine
Tm..... Midpoint at which DNA melts
Tris ..... Tris(hydroxymethyl)aminomethane
TRUCC..... Thermophile Research Unit Culture Collection
Tw ..... Twist
Wr..... Writhe
YE..... Yeast extract
1D..... One dimensional
2D..... Two dimensional

CHAPTER ONE

INTRODUCTION

Preamble

This project was undertaken in order to further characterize and determine the distribution of a unique topoisomerase called reverse gyrase which had just been discovered at the time the project was initiated (Kikuchi and Asai, 1984). The purpose of this study stemmed from a desire to further understand the mechanisms by which extremely thermophilic micro-organisms maintain the integrity of their genetic material. Purified DNA at the temperatures for optimal growth of extremely thermophilic microorganisms (75-105°C) is subject to denaturation (DeLey *et al.*, 1970) (see section 1.6), therefore these organisms must have strategies for dealing with this problem. The binding of proteins to DNA is one obvious way in which thermal denaturation of DNA can be overcome (Reddy and Suryanarayana, 1988). Another, and more subtle strategy, is the introduction of positively supercoiled DNA which resists thermal denaturation to an extent not seen in relaxed or negatively supercoiled DNA (Slesarev and Kozyavkin, 1990). Positive supercoiling of DNA can be catalysed by reverse gyrase *in vitro* but it is not known whether this occurs to the chromosome *in vivo* in organisms which contain this enzyme. A major part of this project was therefore spent in an attempt to establish the supercoiled state of these chromosomes. A positively supercoiled chromosome (all chromosomes so far examined have been negatively supercoiled, Bauer *et al.*, 1980) would not only contribute towards thermal stability of DNA but would also affect gene regulation and transcription from such DNA. Such effects are of intrinsic interest and merit further study though this is beyond the scope of this project.

1.1 DNA Topology and Supercoiling

DNA supercoiling, which is the writhing of a duplex circular DNA molecule about itself, plays a central role in functions involving the bacterial chromosome (Drlica *et al.*, 1984). In fact virtually every physical, chemical, and biological property of DNA including its transcription, hydrodynamic behaviour, energetics, and enzymology are affected by the deformation associated with supercoiling (Bauer *et al.*, 1980). The basic parameters characterising supercoiled DNA are the writhing number (*Wr*), linking number (*Lk*), and the total twist (*Tw*). These are related by the equation:

$$Wr = Lk - Tw \quad \text{(Crick, 1976)}$$

Wr is defined as the contortion of the axis of the double helix in space (Gellert, 1981). Therefore if the axis of the double helix lies entirely on one plane then $Wr = 0$. *Wr* is also referred to as the supercoiling of a DNA molecule.

Lk is defined as the number of times one strand of the double helix passes over the other in a closed circular DNA molecule which is constrained to lie on a plane (Drlica, 1984). *Lk* remains invarient unless one of the strands is broken. Figure 1.1.1 shows a simple double helix with a *Lk* of 4. This is easily determined by following one of the strands and observing how many times it winds around the other strand. Note that because this molecule lies on a plane $Wr = 0$ and *Tw* is equivalent to *Lk.*

Tw is defined as the total number of helical turns found between two strands of a double helical DNA molecule in its native conformation (Drlica, 1984).

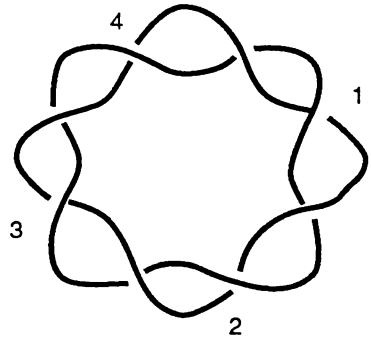


Figure 1.1.1 Closed circular double helix with a linking number of 4.

It is important to note that *Lk* remains invariant, and an integer, in any covalently closed circular DNA (cccDNA) unless one of the DNA strands breaks (Wang, 1983). However *Wr* and *Tw* can and do change with changes in environmental conditions or with binding of proteins or drugs to DNA. When cccDNA is deformed by environmental conditions or by protein or drug interactions there is a free partitioning of the energy of deformation between the writhe and twist elements of the DNA such that the DNA molecule introduces the smallest amount of writhing whilst minimising the twisting component of the DNA molecule (Bauer *et al.*, 1980). Another important point is that supercoiled DNA has a greater free energy than relaxed DNA so that supercoiling is spontaneously lost whenever a nick occurs in the DNA. It has been noted that under physiological conditions the energy associated with supercoiled DNA increases to the square of the superhelical density of DNA (Wang, 1988).

By definition when *Wr* is positive a closed circular DNA molecule is coiled in a left handed manner producing positive supercoils. Conversely when *Wr* is negative a closed circular DNA molecule is coiled in a right handed manner producing negative supercoils (Gellert, 1981) (figure 1.1.2).

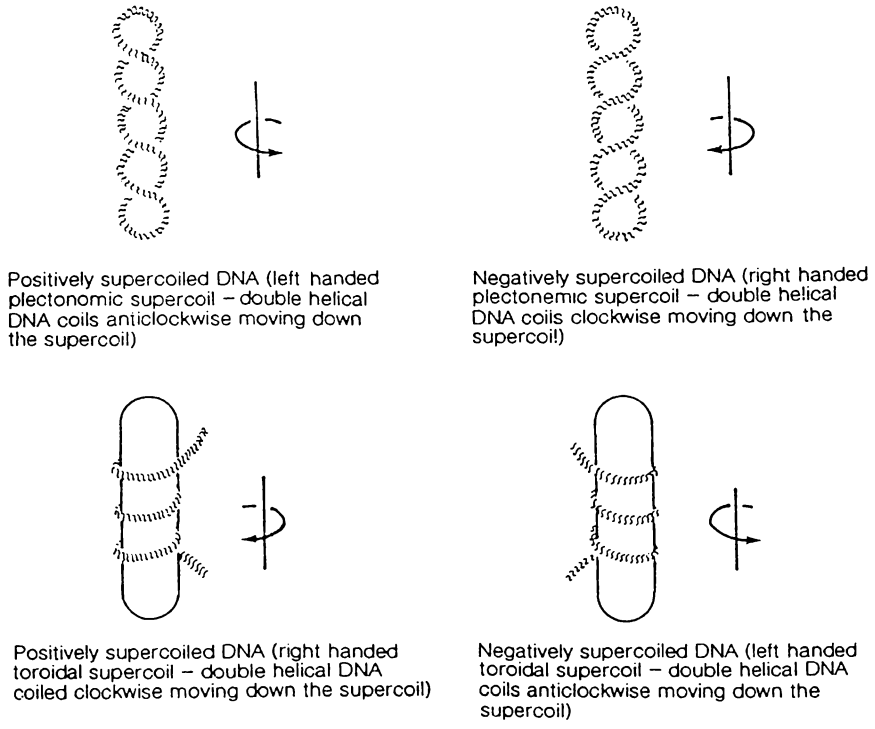


Figure 1.1.2 Free and restrained supercoiling of DNA. A feature of both plectonemic and toroidal supercoiling is that regardless of the position from which the supercoil is viewed the handedness of the supercoil remains invarient. For example if the above positive plectonemic supercoil is viewed upsidedown than a left handed positive supercoil, in which the double helical DNA coils down in an anti-clockwise manner, is still seen.

There are basically two types of supercoiling in living cells. The first type, which is a major component of eubacterial supercoiling, consists of plectonemic supercoils found in closed circular or topologically restrained DNA. This conformation is under torsional strain. The second type occurs in the bulk of chromatin of eukaryotic cells and also in bacteria. In this conformation the DNA is supercoiled around protein, forming a left handed toroidal superhelix. This supercoiled DNA is stabilized by the presence of the protein so that little or no torsional strain is produced in the wrapped DNA (Vosberg, 1985).

Supercoiling of DNA not bound to proteins is always seen to be plectonemic but when DNA is wrapped around a DNA binding protein or proteins (such as HU or histones) then toroidal supercoiling around the protein occurs (Drlica and Rouvière-Yaniv, 1987) (figure 1.1.2). An interesting feature of toroidal supercoiling is that production of negative supercoils requires left handed toroidal supercoils, this is in contrast to the production of negative supercoils in free DNA which displays right handed plectonemic supercoils (Wasserman and Cozzarelli, 1986; Drlica and Rouvière-Yaniv, 1987). Positive supercoiling shows similar characteristics with respect to toroidal and plectonemic supercoils which must be of opposite sign (right and left-handed respectively) to produce positive supercoiling.

When a DNA binding protein is bound to cccDNA in a toroidal manner then the supercoils produced by this attachment must be alleviated by writhing of the DNA along the parts of the DNA molecule which are not attached to the binding protein. For the formula $Wr = Lk - Tw$ to remain true then supercoiling of the opposite sign to that produced by the DNA-protein interaction must be displayed in the free DNA (Gellert, 1981). For example DNA wrapped in a left handed toroid around a binding protein produces constrained negative supercoils ($Wr < 0$) of DNA around the protein. This is alleviated by positive (left handed plectonemic) supercoiling ($Wr > 0$) of the DNA region not bound to protein (figure 1.1.3). Under *in vivo* conditions supercoils found in DNA not bound to protein are subject to action by topoisomerases which in eukaryotes act to relax positive plectonemic supercoils by reducing the linking number (Gellert, 1981). For eukaryotes this results in relaxed DNA which contains negative supercoils restrained by nucleosomes (Gellert, 1981). In eubacteria the situation is complicated by a topoisomerase (DNA gyrase) which can reduce the linking number even when this is energetically unfavourable

(using ATP as an energy source). This results in net negative supercoiling not only from restrained negative supercoils wrapped around HU, and possibly other binding proteins, but also for free DNA not bound to proteins (Drlica and Rouvière-Yaniv, 1987). See section 1.4 for details.

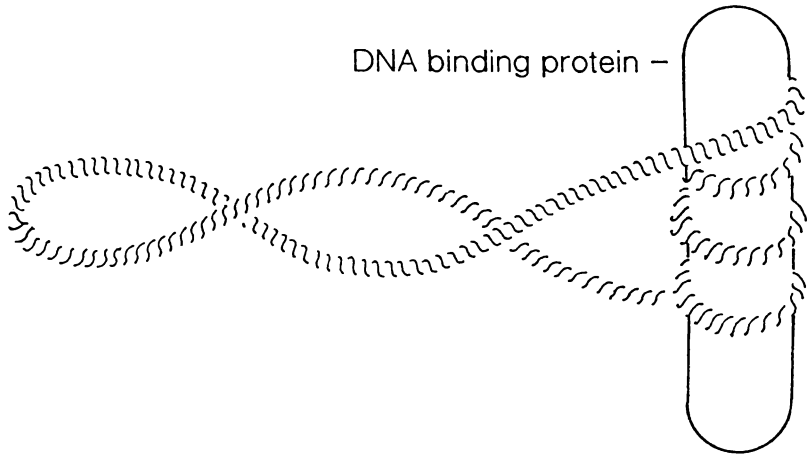


Figure 1.1.3 Negative supercoiling (left handed toroid) of DNA around a binding protein is alleviated by positive supercoiling (left handed plectonemic) in DNA not bound to protein.

The linking number of a duplex ring free of torsional strain (*LK°*) is often used as a reference parameter. This relaxed state is defined as one for which the linking number remains unchanged even if nicks are introduced into the molecule (Wang *et al.*, 1983). For any DNA molecule with a given number of base pairs, *Lk°* is always fixed provided that the ionic strength, temperature, and pH of its environment remain constant (Wang *et al.*, 1983). *In vivo*, various intracellular components will also affect *Lk°*, for example the DNA binding protein HU will reduce the linking number of relaxed DNA (Giaever *et al.*, 1988; Rouvière-Yaniv and Yaniv, 1979). Relaxed DNA under physiological conditions *in vitro* contains 10.5 base pairs per helical turn (Wang, 1982). Therefore by dividing the number of base pairs contained in circular duplex DNA by 10.5 the linking number *Lk°* of relaxed DNA can be found.

A change in *Lk* affects both *Wr* and *Tw* but neither *Wr* or *Tw* can be readily measured experimentally for supercoiled DNA (Wang, 1983). Therefore in experimental situations a different term is used called the linking difference (τ). τ is determined by the equation:

$$\tau = LK - LK^{\circ} \quad \text{(Vinograd } et\ al.\text{, 1968)}$$

Note that τ and *Wr* are not the same, in fact τ contains components of both *Wr* and *Tw* (Gellert, 1981). The usefulness of τ lies in the fact that it can be readily determined by

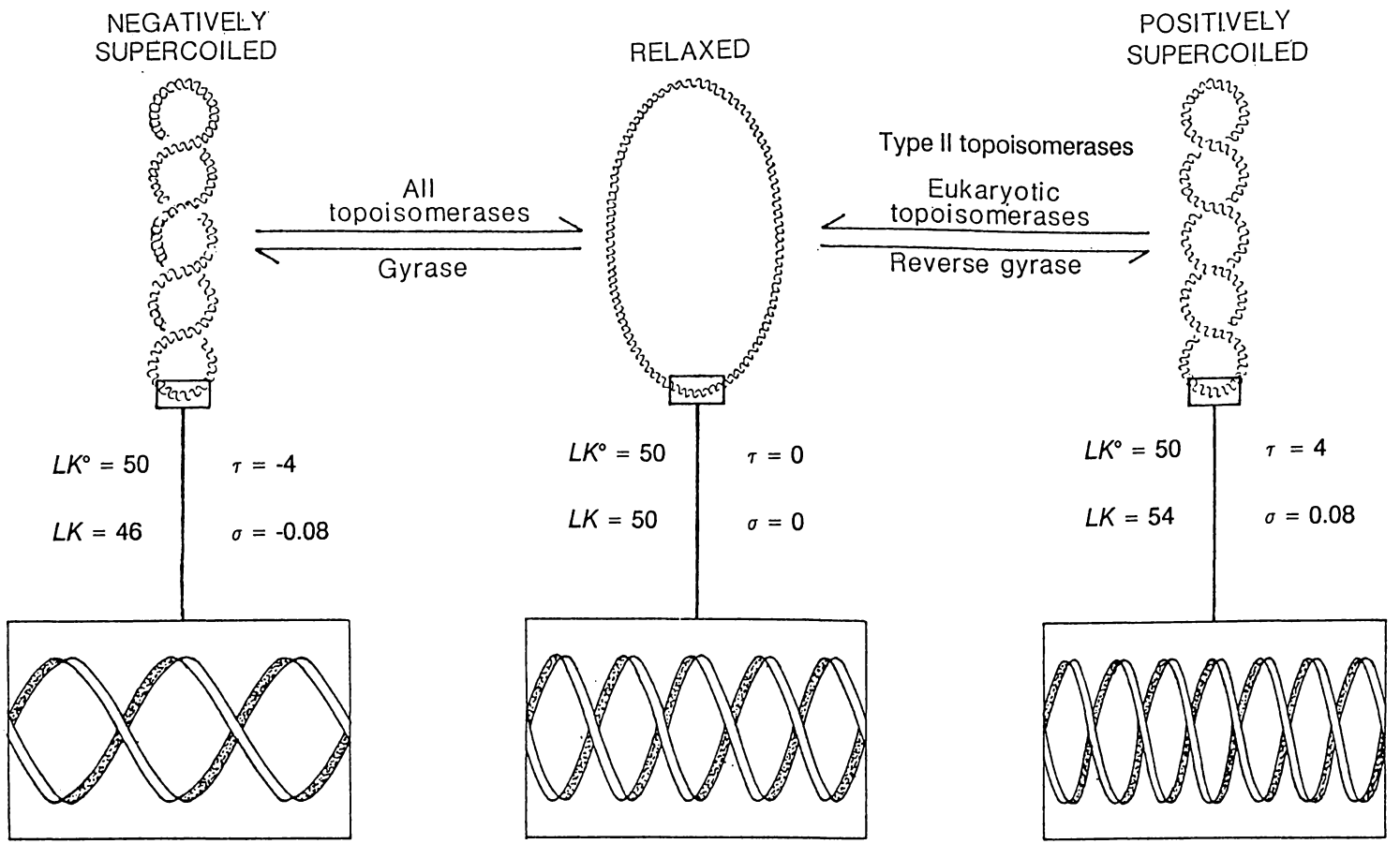


Figure 1.1.4 Supercoiling in covalently closed circular DNA.

experiment (Wang *et al.*, 1983).

If either more or less helical turns are added to a relaxed closed circular DNA molecule, by strand rotation, the linking number *LK* changes. Supercoiling of the DNA molecule then occurs as the DNA is under strain which is minimised by the DNA molecule writhing or supercoiling about itself. Figure 1.1.4 shows an example of the difference in linking numbers and superhelical density that can be found upon negative or positive supercoiling of DNA.

The supercoils formed by DNA with fewer helical turns than in a corresponding relaxed molecule ($Lk < Lk°$) are said to be negatively supercoiled. Supercoils formed by DNA with more helical turns than in the relaxed state ($Lk > Lk°$) are said to be positively supercoiled (Fisher, 1984).

A more useful term than linking difference (τ) is normally referred to when describing supercoiling of DNA. This is called the superhelical density (σ) and it is defined as the number of superhelical turns in a circular duplex molecule per 10 base pairs (Wang, 1985). Superhelical density is of greater practical significance than linking difference as the supercoiling of DNA molecules differing in size can be directly compared. Superhelical density is determined by the equation:

$$\sigma = \frac{Lk - Lk°}{Lk°}$$ (Giaever *et al.*, 1988)

Supercoiling occurs in discrete steps. For example if one helical turn is removed from relaxed circular DNA a change in the linking number of one occurs. One negative supercoil is produced with the open circle of relaxed DNA folding once about itself to produce a figure of eight shape. Subtraction of another helical turn produces a second supercoil and so on. By adding or taking away helical turns, closed DNA molecules of identical sequence may have different linking numbers so producing topological isomers or topoisomers of the DNA molecules (Alberts (ed), 1983). Any change in the linking number requires at least one strand to be broken. If rotation of the strands occur then the topological isomer of DNA is transformed into a different topoisomer. Topoisomerases are thus enzymes which have the ability to alter the topological state of a DNA molecule by utilizing strand breakage, transfer, and rejoining (Wang, 1985).

1.1.1 Structural transitions associated with DNA supercoiling

The stresses associated with negative supercoiling can drive cruciform and Z-DNA formation from B-DNA with the formation of these structures relieving a certain proportion of the stress associated with supercoiling (Rahmouni and Wells, 1989). Cruciform DNA differs from B-DNA by the formation of a region where intrastrand base pairing replaces interstrand base pairing to produce a cruciform shaped structure while Z-DNA differs from B-DNA by existing in a left-handed rather than a right-handed helical form and containing 12 rather than 10.5 nucleotides per helical turn. Both cruciform and B- to Z-DNA formation reduce the intrastrand twist number (*Tw*) though for a DNA segment of a given length the reduction in twist number for the B- to Z-transition is about twice as large as the reduction for cruciform formation (Wang *et al.*, 1983). This may explain why, under physiological conditions, it appears that the formation of Z-DNA is more likely to occur than the formation of cruciform structures (Benham, 1982). Cruciform formation from palindromic sequences *in vitro* have been reported at superhelix densities of -0.04 (Giaever *et al.*, 1988) and -0.06 (Brahms *et al.*, 1989). *In vivo* cruciform formation has also been implicated for a palindromic sequence in the plasmid Col E1 (Panayotatos and Fontaine, 1987).

Z-DNA forms preferentially in regions of alternating purine-pyridimine sequences (Rich *et al.*, 1984; Thomas and Strobl, 1988); the longer the sequence the more favourable is B to Z transition with a minimum length of 7 base pairs being necessary for this transition to occur (Kadalayil *et al.*, 1988). In pBR322, which contains 5 short sequences of B-DNA with the potential to form Z-DNA, a superhelix density of -0.09 is needed before B to Z transition occurs (Brahms *et al.*, 1989). Longer sequences with potential to form Z-DNA have been examined *in vivo* and the B to Z transitions in these cases were reported to occur at a superhelical density of -0.025 (Zacharias *et al.*, 1988). Wildtype *E. coli* has a chromosome sequence with the potential to form Z-DNA (Peck and Wang, 1985). Under normal growth conditions this sequence exists as B-DNA but when negative supercoiling is increased by reducing the level of DNA topoisomerase I in the cell the sequence can be driven into the Z-DNA form (Giaever *et al.*, 1988). The formation of Z-DNA is influenced not only by the supercoiled state of the DNA but also by environmental conditions. Increasing cation concentration for example is known to shift the B- to Z-DNA equilibrium in favour of Z-DNA (Rich *et al.*, 1984). This effect is especially noticeable with

polyvalent cations such as polyamines (spermine and spermidine) which have charges of +4 or +3 (Rich *et al.*, 1984).

A third mechanism which could be used to alleviate the stress associated with negative supercoiling is unpairing and disruption of the double helix. However this apparently does not occur even at negative superhelical densities as high as -0.18 (Brahms *et al.*, 1989).

For eubacteria *in vivo* the positive free energy associated with negative supercoiling is invaluable to the cell by making processes such as replication and transcription, which unwind DNA, energetically favourable.

1.2 DNA topoisomerases

1.2.1 Introduction

DNA topoisomerases are enzymes that control and modify the topological state of DNA. These enzymes produce interconversion between different topological states of a circular DNA molecule by transiently breaking a DNA strand or strands and thereby altering the linking number of the DNA molecule. They also catalyse other reactions such as catenation, decatenation, knotting, and unknotting of DNA molecules. Topoisomerases are essentially involved in a number of vital biological functions, including replication, transcription, and recombination (Wang, 1985). They are ubiquitous enzymes that have been demonstrated in a wide variety of eubacterial, archaebacterial, and eukaryotic species (Pedrini and Ciarrocchi, 1983).

Topoisomerases were first investigated in response to the problem of strand separation during DNA replication. In 1969 it was reported that an activity in *E. coli* extracts was capable of relaxing supercoiled DNA (Wang, 1969). Subsequent purification of this activity produced an enzyme capable of relaxing negatively supercoiled DNA. This enzyme, originally called *w* protein (Wang, 1971) has been renamed *E. coli* DNA topoisomerase I (Wang, 1985). Not long after, in 1972, an activity from mouse cell extract capable of relaxing both positively and negatively supercoiled DNA in the absence of ATP was discovered (Champoux, 1972) (see section 1.1 for definitions of positive and negative supercoiling). It transpired that both the bacterial and eukaryotic enzymes belong to a class of topoisomerases classified as the type I topoisomerases

(section 1.2.2 defines type I and type II topoisomerases).

In 1976 an entirely new topoisomerase, DNA gyrase, was discovered (Gellert *et al.*, 1976). This enzyme negatively supercoils DNA in an ATP-dependent fashion. DNA gyrase was found to belong to a second class of topoisomerases called type II topoisomerases. Another type II enzyme, the DNA topoisomerase of phage T4 was found in 1979 (Liu *et al.*, 1979). This enzyme is ATP dependent but cannot supercoil DNA, however it can catalyse the relaxation of both negatively and positively supercoiled DNA. Subsequently type II topoisomerases were discovered in eukaryotic cells where, it is thought, they form part of the chromatin structure of DNA (Roca and Mezquita, 1989).

Until recently all topoisomerases discovered have fitted comfortably within the framework of their classification system but the discovery of a unique topoisomerase in the archaebacterium *Sulfolobus acidocaldarius* (Kikuchi and Asai, 1984) has somewhat upset this. This enzyme called "reverse gyrase", although initially classified as a type II topoisomerase (Kikuchi and Asai, 1984), was subsequently discovered to be a type I topoisomerase but with several unique features never before found in this class including ATP dependence and supercoiling of DNA molecules (Kikuchi *et al.*, 1986).

1.2.2 Topoisomerases and their distribution

Topoisomerases catalyse the concerted breakage and reunion of DNA strands. The energy of the broken strand or strands is conserved in a covalent linkage with the enzyme to DNA. This allows the break to be resealed in the absence of an external energy source (Dean *et al.*, 1983). Topoisomerases carefully control the breakage and reunion process, never dissociating from the DNA while it is broken.

There are two classes of topoisomerases. Type I topoisomerases are defined by their ability to break only one DNA strand at a time (Wang, 1982). This results in a linking number change of one for each cycle of nicking and sealing of the DNA strand (Srivenugopal, 1984). Type I enzymes are known to catalyse four types of DNA topoisomerization reactions (Figure 1.2.1):

(1) The relaxation of supercoiled DNA (Wang, 1971).

(2) The interconversion of simple and knotted single-stranded DNA rings (Liu *et al.*, 1976).

(3) The linking of a pair of single-stranded DNA rings of complementary sequences into a covalently closed double-stranded ring (Kirkegaard and Wang, 1985).

(4) The catenation of a pair of double-stranded rings provided at least one of the rings contains a pre-existing single-strand nick (Brown and Cozzarelli, 1981).

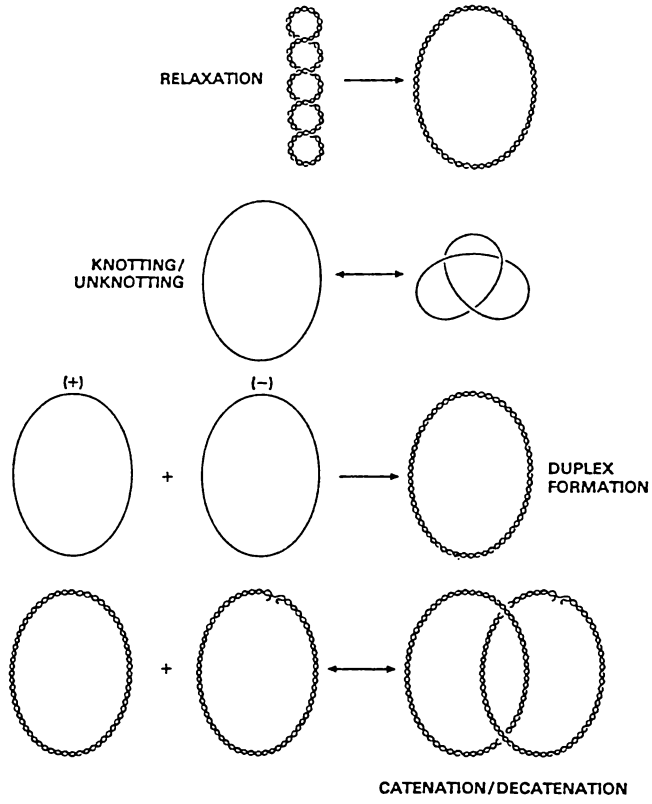


Figure 1.2.1 Reactions of type I topoisomerases (reproduced from Maxwell and Gellert, 1986). The figure shows the major transformations that can be carried out by type I topoisomerases. Note that catenation or decatenation of circular duplexes will occur only if one of the DNA molecules bears a single-stranded break.

The ability of type I topoisomerases to pass single- or double-stranded DNA through a single-stranded break in a DNA molecule explains how these enzymes can carry out the above diverse reactions. A fifth topological reaction, catalysed only by reverse gyrase, involves the positive supercoiling of relaxed or negatively supercoiled DNA (Forterre *et al.*, 1985).

Type II topoisomerases make a transient double-stranded break and pass duplex DNA through the break. This changes the topological linking number of circular DNA in steps of two (Kirchhusen *et al.*, 1985). Topological reactions carried out by type II topoisomerases include (figure 1.2.2):

(1) Relaxation of supercoiled DNA (Cozzarelli, 1980).

(2) For the gyrase enzyme, negative supercoiling of closed circular duplex DNA (Gellert, 1981a).

(3) Knotting and unknotting of duplex DNA rings containing topological knots (Goto and Wang, 1982).

(4) Catenation and decatenation of double-stranded DNA rings (Goto and Wang, 1982).

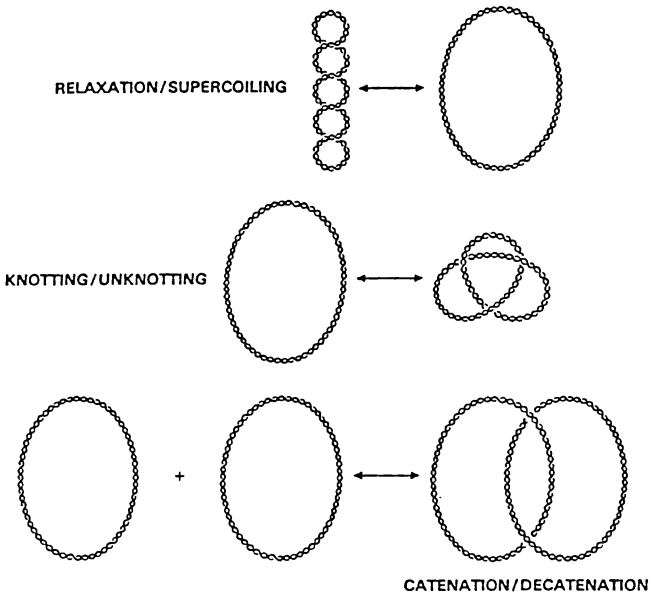


Figure 1.2.2 The reactions of type II topoisomerases (reproduced from Maxwell and Gellert, 1986). The figure shows the major DNA transformations that can be carried out by type II topoisomerases.

The ability of type II topoisomerases to pass double-stranded DNA through a transient double-stranded break in the same or another DNA molecule explains how these enzymes can carry out the above reactions.

From a comparison of topoisomerase genes that have been sequenced it appears that the type II topoisomerases including, eubacterial gyrase, phage T4 topoisomerase II, and eukaryotic topoisomerase II, are evolutionary and structurally related (Lynn *et al.*, 1986). Amino acid sequence similarities suggest that different domains in the eubacterial type II enzymes correspond to domains in eukaryotic type II enzymes (Lynn *et al.*, 1986). It seems probable that the type II subunit in eukaryotes was brought about by joining of the eubacterial gyrase A and B subunits as a consequence of gene fusion (Wang *et al.*, 1987). However DNA gyrase can be distinguished from other type II topoisomerases by characteristics including the conversion of

relaxed or positively supercoiled DNA to a negatively supercoiled form at the expense of ATP hydrolysis (Gellert, 1981b). Other type II topoisomerases can only convert supercoiled DNA to more relaxed forms. A further distinction of DNA gyrase from nearly all other topoisomerases is that cleavage is highly site specific (Fisher *et al.*, 1986) though it may also function in a non-specific manner at replication forks (Drlica, 1980).

In contrast to the sequence similarities of the type II enzymes, eubacterial and eukaryotic DNA topoisomerase I enzymes share no significant amino acid sequence similarities, which is perhaps not surprising considering their mechanistic dissimilarities (Wang, 1987). Differences in mechanism include preference for single-stranded regions by eubacterial topoisomerase I, relaxation of negatively but not positively supercoiled DNA, and covalent protein-DNA linkage at the 5' phosphoryl group of DNA. Eukaryotic topoisomerase I enzymes cleave DNA in double-stranded regions (Been *et al.*, 1984), can relax both negatively and positively supercoiled DNA, and bind covalently to the 3' phosphoryl group of DNA (Prell and Vosberg, 1980).

Table 1.2.1 gives an idea of the distribution and properties of topoisomerases found in living organisms. The relationships amongst topoisomerases shown in this table are described as follows:

(a) Eubacterial type I topoisomerases: These can relax negative supercoils but not positive supercoils. They have a requirement for magnesium ions but ATP is not required for the reaction. All eubacterial type I topoisomerases are single-subunit proteins with a molecular weight of 100,000 to 120,000 daltons (Wang, 1981).

(b) Eubacterial type II topoisomerases: These are all, with one exception, gyrases (Wang, 1985). They require magnesium ions and ATP for the conversion of relaxed or positively supercoiled DNA to a negatively supercoiled form (Peebles *et al.*, 1978). They can, in the absence of ATP, relax negative but not positive supercoils though the rate of relaxation is an order of magnitude lower than the rate of supercoiling (Higgins *et al.*, 1978; Sugino *et al.*, 1977). This enzyme is a tetramer with a total molecular weight of around 380,000 to 420,000 (Gellert, 1981a). The only eubacterial type II topoisomerase without gyrase activity has been found in *E. coli*. This enzyme, called topoisomerase II', cannot negatively supercoil DNA (Gellert 1981a) though, in contrast to DNA gyrase, it can relax positively supercoiled DNA in the absence of ATP

or its analogues. Topoisomerase II' is very similar in structure to DNA gyrase. Both are tetramers and both contain the same A subunit. The B subunit of topoisomerase II' is smaller than the corresponding subunit in DNA gyrase. Using proteolytic digestion it has been shown that the B subunit of both enzymes are similar suggesting that the B subunit of topoisomerase II' is a fragment of the gyrase B subunit (Gellert, 1981b).

(c) Eukaryotic type I topoisomerases: These differ in several respects from eubacterial type I topoisomerases. The eukaryotic enzymes are active in the absence of magnesium and in the presence of chelators, though in many cases the presence of magnesium ions greatly enhances activity. This was shown in the topoisomerase I of *Xenopus laevis* oocytes (Wang, 1985). Eukaryotic type I topoisomerases can relax both positively and negatively supercoiled DNA (Champoux, 1978). ATP is not required for any of these reactions and in some cases actually inhibits activity (Rowe *et al.*, 1981). These enzymes have been purified and appear to be monomeric with a molecular weight of between 60,000 and 120,000. However it has been suggested that some of the preparations containing lower molecular weight fractions are actually proteolytic fragments of larger proteins (Gellert, 1981a). The use of proteinase inhibitors at higher concentrations and rapid fractionation procedures has yielded enzymes with molecular weights around 100,000 (Wang, 1981).

(d) Eukaryotic type II topoisomerases: Unlike their eubacterial counterparts these enzymes relax both positive and negative supercoils. However no eukaryotic type II topoisomerase has been found to produce supercoiling in DNA (Goto and Wang, 1981). All of these enzymes are ATP dependent and require magnesium for activity. The purified enzyme is a dimer consisting of two identical subunits giving a total molecular weight for the complex of about 340kDa (Maxwell and Gellert, 1987).

(e) Archaebacterial topoisomerases: Of the archaebacterial topoisomerases that have been isolated only reverse gyrase has been extensively purified and characterized. This is a unique type I topoisomerase resembling neither eubacterial or eukaryotic topoisomerases. Reverse gyrase positively supercoils DNA and requires ATP for activity (Nakasu and Kikuchi, 1985). It is a single polypeptide with a molecular weight of 120,000 as determined by denaturing gel electrophoresis (Nakasu and Kikuchi, 1985). A second topoisomerase I has been isolated

Table 1.2.1 The distribution and properties of topoisomerases

Enzyme	Type	Requirements		MW (kDa)	Structure	Covalently bound protein-DNA end	Relaxation of supercoils		Supercoiling	References
		ATP	Mg^{2+}				-ve	+ve		
Eubacterial topoisomerase I[a]	I	-	+	~100	monomer	5'	+	-[1]	no	Andera and Mikulík, 1990 Kirkegaard and Wang, 1985 Srivenugopal, *et al.*, 1984 Yamamoto *et al.*, 1985
Eubacterial topoisomerase III[b]	I	-	+	74	monomer	?	+	-	no	Scrivenugopal, *et al.*, 1984
Eubacterial gyrase[c]	II	+	+	100(A) 90(B) 380(total)	A_2B_2	5'	+	+[2]	yes[3]	Bates and Maxwell, 1989 Gellert *et al.*, 1976 Gellert, 1981a
Eubacterial topoisomerase II'[d]	II	+	+	100(A) 50(B) 300(total)	A_2B_2	5'	+	+	no	Brown *et al.*, 1979 Vosberg, 1985
Eukaryotic topoisomerase I[e]	I	-	-[4]	~100	monomer	3'	+	+	no	Carbonera *et al.*, 1988 Gellert, 1981 Vosberg, 1985 Wang, 1985
Eukaryotic topoisomerase II[f]	II	+[5]	+	170(A) 340(total)	dimer	5'	+	+	no	Gellert, 1981 Goto and Wang, 1982
Archaebacterial topoisomerase I[g]	I	-	-	?	?	?	+	-	no	Forterre *et al.*, 1989
Archaebacterial reverse gyrase[h]	I	+	+	~120	monomer	5'	+	-	yes[6]	Collin *et al.*, 1988 Jaxel *et al.*, 1989 Nakasu and Kikuchi, 1985

Enzyme	Type	Requirements		MW (kDa)	Structure	Covalently bound protein-DNA end	Relaxation of supercoils		Supercoiling	References
		ATP	Mg^{2+}				-ve	+ve		
Archaebacterial gyrase[i] [7]	II	?	?	?	?	5'[8]	?	?	?	Forterre *et al.*, 1989 Sioud *et al.*, 1988
Archaebacterial topoisomerase II[j]	II	+	+	60(A) 40(B)	?	?	+	+	?	Kikuchi *et al.*, 1986 Nakasu and Kickuchi, 1985
Viral topoisomerase I[k]	I	-[9]	-	37[10]	monomer	?	+	+	no	Foglesong and Bauer, 1984 Vosberg, 1985
Viral int[l]	I	-	-	40	?	3'	+	+	no	Geider and Hoffmann-Berling, 1981 Maxwell and Gellert, 1987
Viral topoisomerase II[m]	II	+	+	63(A) 52(B) 16(C)[11]	multi subunit	5'	+	+	no	Gellert, 1981a Goto and Wang, 1982 Vosberg, 1985
Mitochondrial topoisomerase I[n]	I	-	-	70[12]	monomer	?	+	+	no	Fairfield *et al.*, 1979 Vosberg, 1985
Chloroplast topoisomerase I[o]	I	-	+	?	?	?	+	-	no	Wang, 1985
Resolvase[p]	I	-	?	21	?	5'	+	?	no	Maxwell and Gellert, 1987

(1) Will act on positively supercoiled DNA in the presence of a region of single stranded DNA (Kiregaard and Wang, 1985).

(2) There is a semantic misunderstanding in many pieces of literature regarding the ability of DNA gyrase to relax positively supercoiled DNA. Many papers state that DNA gyrase

cannot relax positively supercoiled DNA (see for example Gellert, 1981). In fact what occurs is that DNA gyrase, in the pres ence of ATP, not only relaxes positively supercoiled DNA but continues the topological transformation to finally produce a negatively supercoiled DNA product (Brown *et al.*, 1979; Peebles *et al.*, 1978). Both positively supercoiled and relaxed DNA can therefore be negatively supercoiled by DNA gyrase. Furthermore the DNA gyrase enzyme catalyzed relaxation of positive supercoils appears to be an essential element in the relaxation of transriptionally driven positive supercoiling of DNA *in vivo* (Tsao *et al.*, 1989).

(3) Negatively supercoils DNA.

(4) Eukaryotic topoisomerase I enzymes do not require Mg for activity but are normally stimulated by its addition. In one case involving a topoisomerase I from *Xenopus laevis* ovaries, Mg appears to be necessary for activity (Wang, 1985).

(5) A eukaryotic topoisomerase II isolated from Trypanosomes does not appear to require ATP for activity (Douc-Rosy *et al.*, 1986).

(6) Positively supercoils DNA.

(7) Evidence for the existance of DNA gyrase like enzymes in some groups of archaebacteria comes from their sensitivity to doses of coumarins which specifically inhibit DNA gyrase (Sioud *et al.*, 1988).

(8) Indirect proof for 5' attachment comes from the discovery of a protein (presumably DNA gyrase) attached to the 5' end of a DNA strand after treatment with DNA gyrase inhibitors in halobacterial plasmids (Forterre, 1989).

(9) Activity stimulated 2.5x by the addition of 2mM ATP.

(10) The major polypeptide after purification was 37kDa however small amounts of proteins 31, 60, 75, and90 kDa were also observed (Foglesong and Bauer, 1984).

(11) Some preparation of topoisomerase II from phage T4 contain in addition to 63 and 52 kDa polypeptides an extra 16kDa polypeptide (Gellert, 1981a).

(12) It is possible that the mitochondrial topoisomerase I from *X. laevis* oocytes which displayed a molecular weight of ~70 kDa may be a cleavage product and in fact should be 100 kDa in line with classical eukaryotic topoisomerase I enzymes (Vosberg, 1985).

Sources from which topoisomerases have been isolated

(a) *Escherichia coli, Bacillus megaterium, Salmonella typhimurium, Micrococcus luteus, Agrobacterium tumefaciens, Alcaligenes eutrophus, Bacillus stearothermophilus.*

(b) *E. coli*

(c) *E. coli, Bacillus subtilis, M. luteus, Pseudomonas aeruginosa*

(d) *E. coli*

(e) *Drosophila melanogaster*, Rat liver, Human KB cells, Calf thymus, Mouse LA9 cells, Wheat germ, Avian erythrocyte nuclei, *Xenopus laevis*, *Saccharomyces cerevisiae*, Carrot cells, Cauliflower cells, Sea urchin cells

(f) *D. melanogaster*, *S. cerevisiae*, Calf thymus cells, HeLa cells, Trypanosomes

(g) *Thermoplasma acidophilum*

(h) *Sulfolobus acidocaldarius, Desulfurococcus amylolyticus*, Other sulfur metabolizing archaebacteria

(i) *Natronobacterium gregoryi, Methanosarcina barkeri, Halobacterium halobium*

(j) *Sulfolobus acidocaldarius*

(k) Vaccinia virus

(l) Phage λ

(m) Phage T4

(n) Rat liver mitochondria, *X. laevis* mitochondria, Yeast mitochondria

(o) Spinich chlorolplasts

(p) Transposons $\gamma\delta$ and Tn3

from *Thermoplasma acidophilum*. This topoisomerase does not catalyse the supercoiling of DNA but has properties reminiscent of the eubacterial topoisomerase I enzymes though it does not appear to require magnesium for activity (Forterre *et al.*, 1989).

Two archaebacterial type II topoisomerases have been discovered. The enzyme, isolated from *Sulfolobus acidocaldarius*, displays all the characteristics of a classical eukaryotic type II topoisomerase (Kikuchi *et al.*, 1986). However it appears that this enzyme may be a heterodimer consisting of two polypeptides, one of 60,000 daltons and the other of 40,000 daltons. Forterre *et al.*, 1989 have found evidence for a DNA gyrase like enzyme in archaebacteria by showing that the antibiotic coumarins, which specifically inhibit DNA gyrase, affect the growth of various groups of archaebacteria including, halobacteria, methanogens, and *S. acidocaldarius*.

(f) Viral topoisomerases: The Vaccinia virus type I topoisomerase is similar to eukaryotic type I enzymes. It has no magnesium requirement and relaxes both negatively and positively supercoiled DNA (Foglesong and Bauer, 1984). Many viruses produce enzymes that have a topoisomerase action as a secondary activity. Thus enzymes that normally function in DNA strand transfer like the gene A protein of phage ϕX174 and the gene II protein of phage fd can, under certain conditions, catalyse DNA topoisomerization (Meyer and Geider, 1979; Langveld *et al.*, 1980). The λ int gene product which is involved in site-specific recombination can also act as a topoisomerase under certain conditions (Kikuchi and Nash, 1979).

The phage T4 genome codes for a type II topoisomerase which is functionally identical to that of eukaryotic type II topoisomerases. The purified enzyme consists of a multisubunit complex coded for by the T4 genes 39, 52, and 60 with molecular weights of 63, 52, and 16kDa respectively (Liu *et al.*, 1979; Stettler *et al.*, 1979). The small subunit (16kDa) supposedly functions to hold the two larger subunits in juxtaposition within the native protein complex (Seasholtz and Greenburg, 1983). The T4 type II enzyme also displays similar characteristics in structure and properties to the type II topoisomerase isolated by Kikuchi *et al.*, 1986 from *Sulfolobus acidocaldarius*.

(g) Mitochondrial topoisomerases: One of these enzymes has been isolated and partially characterized from rat liver. It has all the properties of a eukaryotic type I topoisomerase

including relaxation of positive and negative supercoils independent of divalent cations (Fairfield *et al.*, 1979). However it is different from these enzymes in being extremely unstable with a half life of four hours at 4°C in its purified form. Its activity is inhibited by the drugs berenil and ethidium bromide at concentrations which are several orders of magnitude lower than those necessary to inhibit the corresponding eukaryotic type I enzyme (Fairfield *et al.*, 1979). However topoisomerase I enzymes isolated from the mitochondria of *Xenopus laevis* oocytes and yeast cells have catalytic and other properties which are apparently identical to those of the corresponding nuclear enzyme, including sensitivity to berenil and ethidium bromide (Brun *et al.*, 1981; Goto *et al.*, 1984).

(h) Chloroplast topoisomerases: A type I topoisomerase has been partially purified from extracts of spinach chloroplasts (Wang, 1985). This enzyme requires magnesium ions and relaxes negatively but not positively supercoiled DNA. In these respects the enzyme resembles eubacterial type I topoisomerases more than the eukaryotic type I enzymes.

1.2.3 Mechanistic Aspects of Topoisomerase Activity

All enzymes which catalyse DNA topoisomerization have several features in common. Firstly, DNA binding and cleavage are required; second, a change in linking number of the DNA molecule by rotation of the cleaved DNA end or ends relative to each other must occur; and last the broken DNA end or ends must be rejoined. Cleavage of DNA in topoisomerases is accompanied by the covalent binding of the enzyme via a tyrosine group to a phosphate group produced by cleavage of a DNA phosphodiester bond. The energy of the broken phosphodiester bond is conserved within the enzyme-DNA complex (Alberts *et al.*, 1983). Type I topoisomerases act by introducing a transient single-stranded break into duplex DNA molecules whereas type II topoisomerases carry out their reactions by introducing breaks on both DNA strands.

The mechanistic properties of eubacterial and eukaryotic type I enzymes differ as do the mechanism of action of type II topoisomerases and these are described in the following sections:

(a) Eubacterial type I topoisomerases: These have been shown by cleavage studies to preferentially interact with a segment of DNA composed of both a single- and a double-stranded region with the site of cleavage falling within the single-stranded region (Wang, 1985). This explains the specificity of the enzyme for negatively supercoiled DNA where it is presumed that

the enzyme binds to DNA causing unpairing of a short region of the DNA duplex (Wang, 1971). Positively supercoiled DNA is not normally a substrate for relaxation by eubacterial topoisomerase type I as binding of the topoisomerase and disruption of a short region of DNA to produce a single-stranded region is energetically unfavourable. However if a single-stranded loop is present then positively supercoiled DNA can be relaxed (Kirkegaard and Wang, 1985).

Eubacterial type I topoisomerases do not have a well defined sequence specificity for DNA. The only rule that has been derived, is that in the majority of cases there is a cytosine base at the fourth position 5' to the cleavage site 5' ----CNNN*---- 3' where N is any base and * is the site of cleavage (Dean *et al.*, 1983). This recognition sequence has also been determined for the archaebacterial topoisomerase type I, reverse gyrase (Kovalsky *et al.*, 1989).

Eubacterial type I enzymes bridge the two ends of the broken strands through covalent and non-covalent interactions with the 3' side and non-covalent interactions with the 5' side. The covalent interaction consists of a linkage between a tyrosine residue of the enzyme and a 5' phosphoryl group of DNA (Depew *et al.*, 1978). Topoisomerase-DNA interaction ensures that the transiently broken DNA ends are kept in close contact by enzyme bridging (Wang, 1985).

As only one DNA strand is cleaved the linking number will change in steps of one as the intact DNA strand rotates 360° about the broken strand. In eubacterial topoisomerase type I enzymes the relaxation of negatively supercoiled DNA shows a strong dependence on the degree of superhelicity of the substrate. Relaxation of weakly supercoiled DNA requires high enzyme concentrations and long incubation times to come to completion.

The topoisomerase may act in two different fashions (distributive or processive) depending on the degree of supercoiling of the DNA substrate and on the salt concentration in which the reaction takes place (Vosberg, 1985). Distributive action involves the dissociation of the enzyme from DNA after each catalytic cycle whereas processive action requires several catalytic cycles to occur before the enzyme dissociates (Maxwell and Gellert, 1987). It appears that under *in vivo* conditions eubacterial topoisomerase type I works in a predominantely distributive fashion (Vosberg, 1985).

A mechanism for the action of eubacterial type I topoisomerases is shown in figure 1.2.3 (Dean *et al.*, 1983).

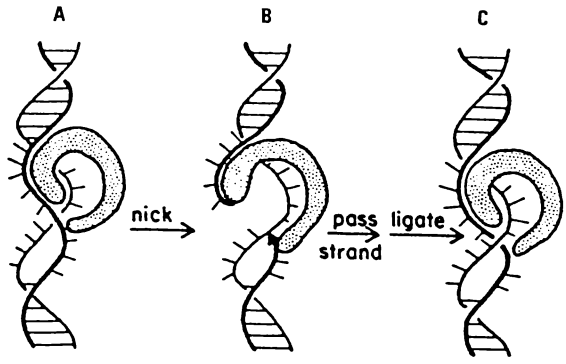


Figure 1.2.3 A one strand sign-inversion mechanism for eubacterial type I topoisomerases (reproduced from Dean *et al.*, 1983). (A) topoisomerase I binds the DNA such that the double helix is unwound and a node is formed by the crossing of the two single strands. (B) the topoisomerase transiently nicks one strand, remains bound to each broken end, and passes the other strand through the break. (C) sealing the break results in a change in linking fixed at +1.

This model of action assumes that the topoisomerase works in a distributive fashion with a cycle of nicking, strand passage, and resealing of the DNA strand occurring for each linking number change of one. An alternative model, called the swivel model, which allows the cleaved DNA to swivel about the long axis of the DNA helix, has also been proposed (Vosberg, 1985). In contrast to the previous model both distributive and processive modes of action would be possible. Of these models the strand passage model best explains the mechanistic details observed for eubacterial topoisomerase type I enzymes (Dean *et al.*, 1983; Maxwell and Gellert, 1987).

(b) Eukaryotic type I topoisomerases: These enzymes preferentially recognise and bind to DNA in duplex regions (Wang, 1985). This explains their ability to relax both positive and negative supercoils. Binding and cleavage of relaxed DNA also occurs (Edwards *et al.*, 1982). No energy cofactor is required for catalysis by these topoisomerases though Mg^{2+} and polycations stimulate activity (Liu, 1989). The chromatin proteins HMG and H1 bind tightly to mammalian topoisomerase I and also stimulate catalytic activity (Javaherian *et al.*, 1983). Eukaryotic topoisomerase type I recognition for DNA shows a preference for a loosely defined 5bp sequence comprised of 4bp to the 5' side of the cleavage site and 1bp to the 3' side (Edwards *et al.*, 1982).

$$5'- \begin{vmatrix} T \\ A \\ C \end{vmatrix} \begin{vmatrix} A \\ C \\ G \end{vmatrix} \begin{vmatrix} T \\ A \\ C \end{vmatrix} \begin{vmatrix} T \\ \\ C \end{vmatrix} * \begin{vmatrix} C \\ \\ A \end{vmatrix} \quad -3'$$

Cleavage occurs at the site indicated by the asterisk. There are two or three alternative bases at each position and the order of preference reads from top to bottom. This consensus sequence is relatively non specific and suggests sequence preference rather than absolute specificity, however a common pattern in all sequences is a change in the local twist angle variation of DNA (Liu, 1989).

Eukaryotic type I topoisomerases are covalently linked by a tyrosine residue to a 3' phosphoryl group on the broken DNA strand (Prell and Vosberg, 1980). The enzyme primarily interacts with DNA over a region covering at least four base pairs 5' to the point of cleavage (i.e. the same side of the break to which the enzyme is covalentely joined) whereas little if any contact occurs with DNA 3' to the transient break (Stevnsner *et al.*, 1989).

It appears that, in contrast to eubacterial type I topoisomerases, enzyme bridging between the two cleaved DNA ends does not occur and the two ends remain in close contact solely due to the double-stranded nature of the DNA around the break (Wang, 1985).

After topoisomerase binding and cleavage of DNA the relative rotation of the two broken ends leads to relaxation of supercoiled DNA. In eukaryotic type I topoisomerases DNA is relaxed to completion with the linking number changing in steps of one. The enzyme may work in both distributive and processive modes depending on the ionic strength of the buffer (McConaughy *et al.*, 1981).

Although the mechanistic details of eukaryotic type I topoisomerases have not been totally elucidated these enzymes are likely to catalyse their reactions using a swivel type mechanism (Vosberg, 1985; Liu, 1989). This is in contrast to the strand passage mechanism postulated for eubacterial type I topoisomerases.

(c) Eubacterial DNA gyrase: The DNA gyrase enzyme is a tetramer composed of 4 subunits in an A_2B_2 configuration. Initial binding to DNA occurs non-covalently at non-random sites (Moore *et al.*, 1983).

Subunit A can be considered as the DNA binding protein in the gyrase complex as the B subunit cannot bind to DNA (Lother *et al.*, 1984). The strength of binding depends on the tertiary structure of the DNA. Gyrase binds less tightly to negatively supercoiled DNA than to relaxed DNA (Cozzarelli, 1980) and not at all to single-stranded DNA (Maxwell and Gellert, 1986). The

binding of the enzyme to DNA causes a segment of ~120 BP to be wrapped around the enzyme in a single positive superhelical turn (Bates and Maxwell 1989).

Cleavage of DNA occurs in both strands near the centre of the wrapped segment generating a four-base stagger between the two cuts. Covalent attachment of a gyrase A subunit to each 5' phosphate end of the cleaved DNA occurs via a tyrosyl-phosphate bond (Tse *et al.*, 1980). Gyrase does not break DNA at a unique invarient sequence, however cleavage is non-random and the following consensus sequence has been proposed based on sequence comparisons (Lockshon and Morris, 1985).

5' - R N N N R N R[T/G]* G R Y C[T/G]Y N Y N[G/T]N Y - 3'

Where N is any nucleotide, R and Y are purine and pyrimidine, respectively, and the asterisk indicates the site of cleavage. This sequence shows elements of dyad symmetry about an axis midway between the cleavage sites in the two strands.

After cleavage a segment of duplex DNA is passed through the double-stranded break. The passage of DNA is in a directional manner that reverses the positive orientation of the DNA segment wrapped around the enzyme (Brown and Cozzarelli, 1979). Two negative supercoils are produced from the reaction and the linking number is changed by steps of two (Cozzarelli, 1980). Finally the broken phosphodiester bonds are reformed. This requires no energy input as bond energy is conserved in the tyrosyl-phosphate linkage (Kirchhausen *et al.*, 1985).

DNA gyrase can act processively on DNA (Morrison *et al.*, 1980). This implies that the enzyme does not dissociate from the DNA between catalytic cycles and that the interactions of the enzyme with DNA must be able to return to their original configuration at the end of each cycle (Maxwell and Gellert, 1986).

ATP is hydrolysed by the B subunits and presumably exerts its effect by changing the conformation of gyrase (Sugino *et al.*, 1978). It has been suggested that the role of ATP is to regenerate the conformational state of the enzyme after a round of supercoiling so that the enzyme can resume another round (Peebles *et al.*, 1978).

Gyrase remains bound at the same specific DNA sites, acting processively through supercoiling cycles until binding is sufficiently weakened by increased supercoiling to release the enzyme (Cozzarelli, 1980). One likely mechanism for gyrase action is shown in figure 1.2.4.

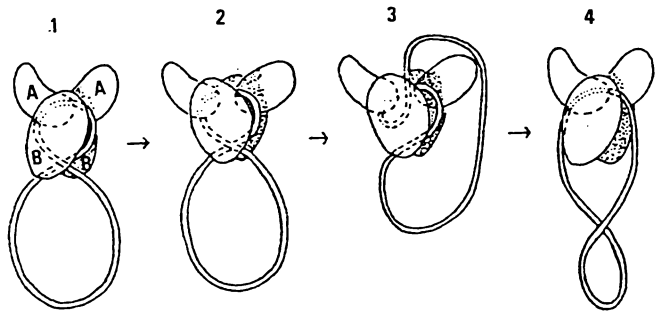


Figure 1.2.4 A possible mechanism of action of DNA gyrase (reproduced from Kirchhausen *et al.*, 1985). The four subunits are labelled in step 1.
(1) DNA is bound to the gyrase A subunits forming a positively supercoiled loop on the gyrase complex. The loop of DNA is shown contained between lobes of the two B subunits. Note that the two A subunits are in close contact at this stage whereas the two B subunits are in an open configuration allowing the insertion of a DNA molecule.
(2) A double-stranded break is made in the DNA, each end of the broken duplex being bound by a gyrase A subunit. The A subunits then move apart leaving a gap between the ends of the broken DNA molecule. The B subunits have moved together thus trapping the DNA loop within the complex.
(3) One of the two arms of the DNA loop, constrained between B subunits and trapped by closure of the B-B contact, diffuses through the double-stranded break.
(4) Completion of the reaction cycle requires rearrangement of the DNA after strand passage and, concomitant with ATP hydrolysis, rearrangement of the subunits with closure of the A-B subunit contact and opening of the B-B subunit contact.

(d) Eukaryotic and T4 type II topoisomerases: These topoisomerases are both structurally and functionally related to DNA gyrase (Liu, 1984). However the differences between these two groups of enzymes is not confined to the catalysis of negatively supercoiled DNA which is unique to DNA gyrase.

Binding of eukaryotic type II topoisomerases, in contrast to DNA gyrase, occurs preferentially with negatively supercoiled DNA over relaxed or nicked DNA (Osheroff *et al.*, 1983). Relaxation of positively and negatively supercoiled DNA occur at a comparable rate. T4 type II topoisomerase, and probably eukaryotic type II topoisomerases, bind tightly to and cleave single-stranded DNA (Kreuzer, 1984). This DNA is also a potent inhibitor of the relaxation reaction of eukaryotic topoisomerase type II whereas single-stranded DNA has no effect on the supercoiling reaction of gyrase (Vosberg, 1985). The cleavage of DNA by eukaryotic type II topoisomerases is similar to gyrase. Cleavage of the two strands is staggered by four base pairs and a covalent tyrosine-phosphate bond is formed on the 5' phosphoryl end of each broken strand (Sander and Hsieh, 1983). DNA cleavage by these enzymes is relatively non-specific, however for the T4 enzyme it has been suggested that cleavage occurs at or near the base of hairpins (Kreuzer,

1984). A consensus sequence for the *Drosophila* type II topoisomerase has also been published (Maxwell and Gellert, 1985).

5' - G T N |A| A Y * A T T N A T N N G - 3'
|T|

Eukaryotic type II topoisomerases catalyse the catenation/decatenation and knotting/unknotting of circular duplex DNA molecules (Tse and Wang, 1980). Therefore the mechanism of these enzymes (like gyrase) involves duplex DNA passing through the double-stranded break made by the enzyme. This is confirmed by relaxation of positively and negatively supercoiled DNA which occurs in steps of two. These topoisomerases work processively (Osheroff *et al.*, 1983) so the enzyme must remain bound to DNA between catalytic cycles and return to its original configuration at the end of a cycle. These enzymes can be made to work in a distributive mode by increasing the ionic strength of the buffer (Osheroff *et al.*, 1983).

Eukaryotic type II topoisomerases require ATP for catalysis of reactions even when these are energetically favourable (Liu *et al.*, 1979). Like DNA gyrase, it appears that they require the binding of ATP for DNA strand passage and hydrolysis of ATP to restore the enzyme to its original conformation ready for a new cycle of catalysis (Osheroff *et al.*, 1983).

Mechanistically it appears that all type II topoisomerases operate in a similar manner. However, unlike DNA gyrase, other type II enzymes cannot wrap DNA with a positive twist and so cannot supercoil circular DNA molecules (Gellert, 1981a). Without this orientating factor, DNA would more frequently pass through a transient break with a sense determined by the supercoiling of the DNA, thus relaxing any supercoils.

Recently an alternative mechanism of action for eukaryotic type II topoisomerases was described (Pommier *et al.*, 1989). Here the enzyme, described as a "donut" complex, non-covalently attachs to and moves along a DNA complex. This movement is retarded by polyamines and possibly by alternative secondary DNA structures. Figure 1.2.5 shows details of this model.

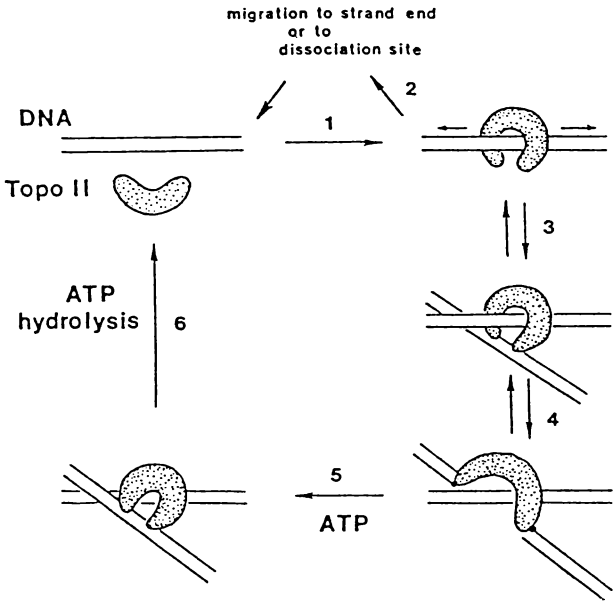


Figure 1.2.5 A model for the strand passage process by eukaryotic type II topoisomerases (Reproduced from Pommier *et al.*, 1989).
(1) The enzyme wraps around a DNA double helix to form a non-covalent "donut" complex which can migrate along the DNA.
(2) If migration leads to a DNA end then dissociation will occur. If a specific dissociaton site is encountered then dissociation can occur at that site with low probability.
(3) The enzyme interacts reversably with a second DNA strand segment which becomes bound to the two cleavage sites of the enzyme.
(4) The DNA strands are cleaved and the 5' strand termini are covalently linked to the enzyme.
(5) ATP binds to the enzyme forcing strand passage and closure of the DNA ends.
(6) ATP hydrolysis regenerates the enzyme.

1.2.4 Biological Roles of Topoisomerases

Topoisomerases are vitally involved in nearly every aspect of chromosome activity. This includes replication, transcription, and recombination (Drlica, 1984). Topoisomerase activity may be direct as in the coupled breakage and rejoining of DNA strands in recombination, or indirect through their effect on the supercoiled state of DNA (Gellert, 1981).

(a) The Role of Toposiomerase activity in Replication: There is convincing evidence that topoisomerases, especially DNA gyrase, are essential for replication of circular duplex DNA in bacteria. This evidence comes from two lines of research: Firstly drugs, notably novobiocin and coumermycin, which block gyrase activity, prevent replication of bacterial cells. Secondly

temperature sensitive mutations in either of the two genes coding for gyrase subunits preclude cellular DNA replication (Gellert, 1981b). Replication can be divided into three areas: initiation of replication; elongation phase of replication; and termination of replication. Topoisomerases appear to play a role in all three phases of replication.

Initiation of replication: DNA gyrase is required for the initiation of replication (McCarthy, 1979; Kreuzer and Cozzarelli, 1979). This has been found to be true not only in *in vivo* studies but also in *in vitro* studies with purified components (LeBowitz *et al.*, 1985). Apparently gyrase works indirectly by providing a negatively supercoiled substrate which is a prerequisite for binding of proteins required to start replication (Drlica, 1984). A temperature sensitive mutuation at the gyrB locus in *E. coli* blocks initiation. Chain elongation however is essentially unchanged. Replication of bacteriophage ϕX174 is initiated by site-specific cleavage using the phage gene A protein. This cleavage event requires the presence of DNA gyrase or alternatively a negatively supercoiled DNA substrate (Marians *et al.*, 1977). In yeasts, and probably other eukaryotic cells, the type II topoisomerase does not seem to be essential for the initiation of replication (Wang, 1987).

Elongation phase of replication: A swivel problem arises during movement of a replication fork. DNA unwinding and strand separation associated with chain elongation generate torsional tension ahead of replication forks in topologically constrained DNA. Without a topoisomerase acting as a swivel the torsional strain eventually becomes so great that fork movement stops (Drlica, 1984). Topoisomerases, including the archaebacterial type II topoisomerase, which can relax positive supercoils, could function in this capacity to relieve the tension caused by chain elongation. In an *in vitro* Phage λ replication system, it has been shown that fork propagation in a circular DNA molecule can occur in the presence of either one of four topoisomerases tested: *E. coli* gyrase, *E. coli* topoisomerase I, mammalian topoisomerase I, and II (Wang, 1987). In eubacteria, gyrase maintains a negative superhelical tension in DNA which eliminates topological barriers to fork movement. In effect, the negative superhelical turns pull the replication fork along (Gellert *et al.*, 1976). One temperature sensitive gyrase mutant rapidly stops replication and is implicated in blocking of chain elongation (Filutowicz and Jonczyk, 1983). Further evidence comes from gyrase inhibitors which block replication at the elongation step. The rapidity of this reaction suggests that DNA gyrase may function directly at

replication forks in addition to its role in maintaining general supercoiling of the chromosome (Drlica *et al.*, 1980). Inhibition by naladixic or oxolinic acid is seen in this model as causing a physical block to fork movement, for example by forming a very stable DNA-enzyme complex (Gellert, 1981a). Eukaryotic cells have a similar requirement for a swivel to relieve the torsional strain generated by fork propagation. Using a cell free system consisting of HeLa cell extract and SV40 T-antigen for the replication of SV40 DNA, it has been demonstrated that both eukaryotic topoisomerase I or II can fulfill the role of a swivel (Yang *et al.*, 1987). A further set of experiments have shown interaction of eukaryotic type II topoisomerases with DNA molecules near DNA replication forks in mammalian cells (Nelson *et al.*, 1986). This would suggest that at least one of their functions *in vivo* is to act as a swivel during replication.

Termination of replication: At the end of a round of replication newly replicated progeny DNA molecules are multiply intertwined or catenated (Sogo *et al.*, 1976). Topoisomerases are implicated in the resolution of these DNA molecules which is necessary for proper chromosome segregation. It has been shown that chromosomes from temperature sensitive DNA gyrase mutants of *E. coli* are obtained as doublets from cells grown under non-permissive conditions. These doublets can be resolved by the addition of gyrase (Steck and Drlica, 1984). *In vivo* studies in yeasts have also provided strong evidence that a type II topoisomerase is involved in the segregation of intertwined DNA pairs (DiNardo *et al.*, 1984). Furthermore the unlinking of catenated DNA networks found in the kinetoplasts of Trypanosomes can be catalysed by DNA gyrase or T4 DNA topoisomerase (Marini *et al.*, 1980).

(b) The Role of Topoisomerase Activity and Supercoiling in Transcription: Evidence has shown that transcription of negatively supercoiled DNA is generally enhanced over that of relaxed, nicked, or linear DNA (Botchan, 1976; Seeburg *et al.*, 1977). This is thermodynamically feasible in light of the fact that binding of RNA polymerase unwinds DNA by almost one turn (Saucier and Wang, 1972). Binding of RNA polymerase to negatively supercoiled DNA is thus aided by a favourable free energy change. In agreement with this are results which demonstrate a positive correlation between DNA supercoiling by gyrase and stimulation of transcription both *in vitro* (Akrigg and Cook, 1980) and *in vivo* (Oostra *et al.*, 1980; Kano *et al.*, 1981). The correlation between transcription and supercoiling is not simple however and various promoter

sites appear to have quite different sensitivities to the degree of supercoiling (Botchan *et al.*, 1973; Sanzey, 1979). For instance the inhibition of DNA gyrase by nalidixic acid in *E. coli* reduced the expression of the maltose and lactose operons by 5-10 fold. However expression of threonine, tryptophane, and tryptophanase loci were almost unchanged (Sanzey, 1979). Apparently the *in vitro* transcription of certain eukaryotic genes can also be modulated by a change in DNA topology (Hirose and Suzuki, 1988)

Supercoiling can have a global effect on certain promotors, for example catabolite-repressable promotors require a high degree of negative supercoiling for expression (Sanzey, 1979). An increase in supercoiling also appears to activate genes which are important in the osmoprotection of bacteria (Higgins *et al.*, 1988). It is perhaps not surprising that the transcription of eubacterial topoisomerases are regulated by a change in supercoiling; thus transcription from the promoters of *E. coli* gyrA and gyrB genes was shown to be more efficient when the DNA template was more relaxed (Menzel and Gellert, 1983) and, in contrast, the transcription of the *E. coli* topoisomerase I gene was shown to be more efficient when the DNA template was more negatively supercoiled (Tse-Dinh and Beran, 1988).

Direct evidence for the influence of supercoiling on transcription can be seen in a cell-free coupled transcription-translation system. For example, with relaxed ColE1 DNA as a template, expression of the colicin gene is strongly depressed by gyrase inhibitors. If ColE1 DNA is added in a supercoiled form normal expression occurs. The need for a gyrase function can therefore be bypassed if the DNA is already supercoiled (Gellert, 1981b).

Apart from the thermodynamically favourable binding of RNA polymerase to negatively supercoiled DNA there are a number of other ways in which supercoiling could effect transcription. For example transcription can be blocked *in vitro* by increased supercoiling leading to conformational changes in DNA producing Z-DNA or cruciforms, though whether this occurs *in vivo* is not known (Brahms *et al.*, 1985; Pruss and Drlica, 1989). Supercoiling induced deformation of some promoters may be required for recognition by RNA polymerases. Furthermore DNA looping, which can be induced by supercoiling, has been implicated in the regulation of gene expression (Pruss and Drlica, 1989). Indirect evidence that eukaryotic type II topoisomerases have an effect in transcriptional control has come from studies which show that

this topoisomerase recognises DNA sites mainly in intergenic regions (Udvardy *et al.*, 1985).

Topoisomerases in both eubacteria and eukaryotes are likely to be involved in relieving superhelical tension associated with movement of the transcription complex along the DNA. A model to explain the effect of transcription on supercoiling and the role of topoisomerases in this has been proposed (Wu *et al.*, 1988). This is dealt with in detail in section 1.5.

(c) Topoisomerases in DNA Recombination and Repair: Topoisomerases have been implicated in recombination events and in fact DNA gyrase was first discovered as a factor needed to activate DNA for the phage lambda integrative recombination reaction *in vitro* (Mizuuchi *et al.*, 1979). There are three biochemical characteristics of topoisomerases which indicate their involvement in recombination:

(1) Type I topoisomerases permit intertwining of circular complementary strands (Champoux, 1977). Experiments suggest a plausible role for topoisomerases in the pairing of strands undergoing recombination (Wu *et al.*, 1983).

(2) The ability of a topoisomerase to break and rejoin DNA strands, and to carry out intermolecular strand transfer in some cases, suggests that topoisomerases could catalyze strand breakage and rejoining in DNA recombination (Wang, 1985).

(3) In prokaryotes the role of topoisomerases in maintaining the supercoiled state of DNA may in turn affect certain recombination pathways (Drlica, 1984).

Generalized recombination can be enhanced by the use of DNA gyrase to form a negatively supercoiled substrate. For example, recombination between single-stranded fragments of ϕX174 DNA and circular duplex ϕX174 occurs at higher frequencies if the circular ϕX174 is supercoiled (Holloman and Radding, 1976).

Site specific recombination involving the integration and excision of bacteriophage lambda, and transposons, include recombination events in which two short homologous DNA sequences pair, break, and rejoin. This process is mediated by proteins, some of which exhibit topoisomerase activity, for example, the int protein of phage λ and the resolvase of transposons Tn3 and $\gamma\delta$ (Drlica, 1984; Vosberg, 1985). Furthermore this reaction normally requires a negatively supercoiled DNA substrate (Mizuuchi *et al.*, 1978).

Illegitimate recombination mediated by DNA gyrase has been implicated in some cases.

This reaction is stimulated ten fold by oxolinic acid, a drug which stabilizes the DNA-gyrA reaction intermediate (Ikeda *et al.*, 1981). It is suggested that gyrase participates in illegitimate recombination by a mechanism involving gyrase subunit exchange. Exchange of gyrA protein-DNA fragments between two gyrase enzymes would lead to a recombination event (Ikeda *et al.*, 1982; Marvo *et al.*, 1983).

Relationships between DNA repair and supercoiling have been investigated by using mutations which alter cellular levels of supercoiling but so far no simple correlation has emerged (Drlica, 1984). However, in some cases at least, supercoiling is found to stimulate repair of DNA damage in UV-irradiated cells (Hays and Boehmer, 1978; von Wright and Bridges, 1981).

(d) Topoisomerases and DNA chromatin: Eukaryotic type II topoisomerases have been shown to be a prominant component of the nuclear scaffold or matrix (Earnshaw *et al.*, 1985; Gasser and Laemmli, 1986; Newport, 1987) and appear to play a critical role in nucleosome formation *in vivo* and also *in vitro* (Ryoji and Worcel, 1984; Glikin *et al.*, 1984). Topoisomerase II may be located at specific sequences at the base of a chromosome loop (Mirkovitch *et al.*, 1984) which suggests that it has a functional role in determining the superhelical strain of these loops (Weintraub, 1985). These loops could therefore represent units of superhelical domains perhaps similar in this respect to the domains found in eubacterial chromosomes (Cook and Brazell, 1978).

It is thought that in eubacteria DNA gyrase may also be involved in the formation of DNA-protein scaffolds and form part of the complex located at the base of chromosome domains (Yang and Ames, 1988).

1.3 Reverse gyrase

1.3.1 Characteristics of reverse gyrase

Reverse gyrase is a type I topoisomerase which has the unique property of introducing positive superhelical turns into closed circular DNA (Nakasu and Kikuchi, 1985). Reverse gyrase has been purified and characterised from *Sulfolobus acidocaldarius* (Nakasu and Kikuchi, 1985; Nadal *et al.*, 1988), *Desulfurococcus amylolyticus* (Slesarev, 1988), *Sulfolobus solfataricus* (this

thesis), and TRUCC isolate AN1 (this thesis). All of these enzymes have identical properties with the exception that the reported molecular weights vary from 120 to 135kDa. The enzyme is a monomer in its native state as determined by gel filtration (Nakasu and Kikuchi, 1985) and sucrose gradient sedimentation (Nadal *et al.*, 1988) under non-denaturing conditions. This was confirmed by electron microscopy (Nadal *et al.*, 1988). Reverse gyrase has a slightly acidic isoelectric point (pI5.6-5.9) (Nadal *et al.*, 1988) and contains no cysteine residues but contains a large number of hydrophilic residues such as leucine (10.7%) and isoleucine (27.2%) (Nadal *et al.*, 1988).

Temperature effects: Reverse gyrase is active at temperatures ranging from 60°C to 100°C the highest temperature assayed (Nakasu and Kikuchi, 1985; Slesarev, 1988). Both Nakasu and Kikuchi, 1985, and Slesarev, 1988 found optimum enzyme activity at the highest temperature assayed. This could mean that optimum activity may lie above 100°C. An optimum activity for reverse gyrase, or any enzyme, must be accepted with care however, as it reflects a balance between enzyme denaturation over the time of the assay and substrate catalysis. Both enzyme denaturation and activity are likely to increase with increasing temperature and be affected by the incubation period and assay constituents including the presence or absence of substrate.

pH effects: Reverse gyrase is reported to be active in a neutral pH range of 6.5 to 8.5 (Nakasu and Kikuchi, 1985) but whether this takes temperature effects of buffers into account is not stated. Most reactions with reverse gyrase are carried out with Tris/HCl pH7.5-8.0 (25°C) as the buffer which at a temperature of 75°C gives an effective pH optimum of 6.1 to 6.6.

Ionic effects: Reverse gyrase requires salt for activity and both NaCl and KCl are effective. The optimum NaCl concentration for relaxation of negatively supercoiled DNA is in the range of 160-250mM (Nakasu and Kikuchi, 1985; Slesarev, 1988). Under these conditions the enzyme appears to work in a distributive manner (Forterre *et al.*, 1985). Under conditions of lower ionic strength (5-50mM NaCl, (Nakasu and Kikuchi, 1985), or 20-60mM KCl, (Forterre *et al.*, 1985)) the enzyme appears to work in a processive manner with the DNA substrate attaining a higher positive superhelicity than under distributive conditions (Forterre *et al.*, 1985).

Effects of divalent cations: Divalent cations are required for activity with a concentration

of 3-10mM Mg^{2+} demonstrating optimum activity. Ca^{2+} could be substituted but was slightly less effective (Nakasu and Kikuchi, 1985). If magnesium was omitted from the assay, reverse gyrase introduced nicks into DNA when the reaction was stopped by SDS (Nakasu and Kikuchi, 1985).

Effects of nucleotides: ATP, UTP, GTP, and CTP can all be utilised by reverse gyrase to catalyse the relaxation of negatively supercoiled DNA (Shibata *et al.*, 1987). However relaxation was slow with all nucleoside triphosphates except ATP which supported a rapid relaxation and positive supercoiling of cccDNA (Nakasu and Kikuchi, 1985). Only ATP supports the positive supercoiling of cccDNA undoubtedly through the energy released by its hydrolysis. Other nucleoside tri-phosphates could not be hydrolysed by reverse gyrase (Shibata *et al.*, 1987). ATP is required in small concentrations for activity e.g. 10uM ATP is enough to catalyse positive supercoiling (Nakasu and Kikuchi, 1985). This is 30x less than the amount required by type II topoisomerases e.g. 300uM ATP for the type II enzyme in *Sulfolobus* (Nakasu and Kikuchi, 1985). For optimum activity of reverse gyrase an ATP concentration of 0.1-5mM ATP is necessary (Slesarev, 1988).

Effects of drugs: Coumarins (e.g. novobiocin and coumermycin), drugs which specifically inhibit the gyrB subunits of eubacterial DNA gyrase, had little effect on reverse gyrase activity at concentrations which specifically inhibit eubacterial gyrase. This was also true for quinolones (e.g. perfloxacin), drugs which specifically inhibit the gyrA subunit of eubacterial DNA gyrase (Forterre *et al.*, 1985; Nakasu and Kikuchi, 1985). Similarly drugs which inhibit activity of eukaryotic type II topoisomerases (e.g. epipodophyllotoxin) had no effect on reverse gyrase activity (Forterre *et al.*, 1985). Naladixic acid (100ug/ml), which is active against DNA gyrase, was another drug which did not inhibit reverse gyrase activity (Nakasu and Kikuchi, 1985).

Drugs which do have an effect on activity include the non-intercalating drug berenil (200ug/ml) and the DNA intercalating drugs adriamycin (1ug/ml) and ethidium bromide (3ug/ml) (Nakasu and Kikuchi, 1985).

1.3.2 Mechanistic aspects of Reverse gyrase

Reverse gyrase will bind to both double-stranded and single-stranded DNA (Shibata *et al.*, 1987). However reverse gyrase has a higher affinity for single-stranded DNA as measured by

ATPase activity and by competition experiments (Shibata *et al.*, 1987; Slesarev and Kozyavkin, 1989). A preference for single-stranded DNA regions is also found when reverse gyrase is incubated with a positively supercoiled plasmid containing a single-stranded loop. This produces a stimulation of reverse gyrase activity not seen in the positively supercoiled parental plasmid without a single-stranded loop (Slesarev and Kozyavkin, 1989). Reverse gyrase works more efficiently on negatively supercoiled DNA, which facilitates the formation of single-stranded regions, than on relaxed or positively supercoiled DNA (Slesarev, 1988).

The binding of reverse gyrase to DNA is temperature dependant and lack of enzymatic activity at temperatures below 50°C can be attributed to the absence of reverse gyrase-DNA interaction (Jaxel *et al.*, 1989).

Reverse gyrase has a cleavage site specificity which is identical to that found for eubacterial topoisomerase type I enzymes. Cleavage occurs at the sequence, 5'---CNNN*---3', where N is any base and * is the site of cleavage. This pattern held true for 30 out of 31 cleavage sites which were examined (Kovalsky *et al.*, 1989). Covalent binding of reverse gyrase occurs at the 5' residue of the cleaved DNA (Jaxel *et al.*, 1989; Kovalsky *et al.*, 1989).

The effect of stoichiometric amounts of inactive reverse gyrase bound to nicked DNA has been examined by Jaxel *et al.*, 1989. After reverse gyrase binding, covalent closure of DNA by a thermophilic DNA ligase, and removal of bound protein, they discovered that negatively supercoiled DNA was produced with an unwinding value of -0.5 turns per enzyme molecule present (this value was a minimum estimate). The structural change that produces this unwinding is not known, however of three possibilities; protein wrapping around the enzyme, DNA bending or kinking, or DNA untwisting due to enzyme binding, the latter appears most likely.

Reverse gyrase can act in either a distributive or processive manner depending on the ionic conditions in which it is assayed (Forterre *et al.*, 1985). The ability of reverse gyrase to act in a processive manner should mean that it is capable of catalysing a number of relaxation or supercoiling cycles while remaining bound to DNA and that regeneration of the enzyme in order to begin another round of catalysis can occur whilst still bound. In the processive mode it seems likely that catalysis of a DNA substrate continues until a positive superhelicity of DNA is reached such that reverse gyrase dissociates from the DNA.

ATP is hydrolyzed by reverse gyrase and it is likely that both ATP binding and hydrolysis induce structural changes in the enzyme leading to single-strand passage in a directional manner that produces a positively supercoiled product. A model for the action of reverse gyrase is shown in figure 1.3.1.

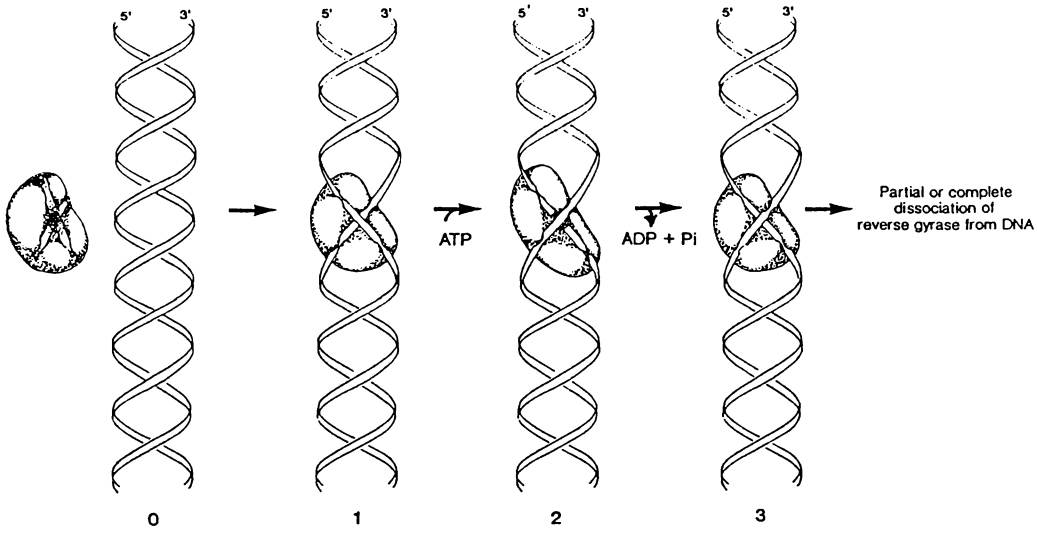


Figure 1.3.1 A model for the directional strand passage mechanism of reverse gyrase (adapted from Slesarev and Kozyavkin, 1990).

(1) Reverse gyrase binds to DNA and in doing so introduces a twist of -0.5 into the DNA helix. The introduction of such a twist is energetically favourable at negative superhelicities and can be compared to the binding of RNA polymerase to negatively supercoiled DNA which is favoured by the introduction of a negative twist.

The initial orientation of the DNA strands on the enzyme is strictly defined and is the opposite to that found in a right handed helix. This orientation could conceivably occur by local untwisting from reverse gyrase binding.

(2) Cleavage of one DNA strand occurs with covalent binding of the enzyme to the 5' group of the nick. ATP is probably bound to the enzyme at this stage and results in a conformational change where the cleaved DNA ends, both bound to the enzyme, are moved apart.

(3) Strand transfer occurs where the intact DNA strand is moved through the broken strand. The broken strand is then religated. It is probably at this stage that ATP is hydrolysed. It may be needed to activate strand passage which is expected to be thermodynamically unfavourable during positive supercoiling. Evidence for a requirement of ATP at this stage comes from the observation that in the absence of ATP reverse gyrase can bind to and nick DNA with covalent binding of the enzyme to a nicked DNA end. However the reaction does not proceed further and upon addition of SDS, nicks are introduced (Nakasu and Kikuchi, 1985).

(4) Partial or complete dissociation of reverse gyrase from the DNA substrate occurs. Partial dissociation is perhaps more likely as the enzyme shows processive activity. A likely candidate for DNA binding between catalytic cycles is the noncovalent interaction at the cytosine residue of the 3' terminus at the cleavage site (Kovalsky *et al.*, 1989).

1.3.3 The relationship of reverse gyrase to other topoisomerases

Reverse gyrase shares many similarities with classical eubacterial type I topoisomerases; they have the same substrate specificity for single-stranded DNA; they have the same cleavage site specificity; they both require magnesium for activity; they both covalently bind to the 5' terminus of the single-stranded break; they both fail to relax positively supercoiled DNA; and their mechanism of action is similar requiring a protein bridged strand passing mechanism. All of these features are unique to eubacterial topoisomerase I and reverse gyrase.

The similarity of reverse gyrase to DNA gyrase is superficial and relates only to their mutual and unique ability to supercoil DNA albeit with a different sign and their hydrolysis of ATP in this process.

Reverse gyrase is unique from eubacterial type I topoisomerases in several respects. Firstly it can hydrolyse ATP to produce the formation of positively supercoiled DNA from a negatively supercoiled or relaxed DNA substrate. Eubacterial type I enzymes, cannot hydrolyse ATP, cannot catalyse the production of positive supercoils, and bind poorly to relaxed DNA (Dean *et al.*, 1982). For reverse gyrase, ATP or another nucleoside triphosphate is required for relaxation of negatively supercoiled DNA. Such a requirement is not found in eubacterial topoisomerase I.

Considering that the enzymatic relaxation of negatively supercoiled DNA and production of positive supercoils are mechanistically identical and require the same strand passage orientation it is tempting to speculate that reverse gyrase is a eubacterial type I enzyme but with an ATPase activity that can force strand passage even under energetically unfavourable conditions.

1.3.4 The distribution of reverse gyrase

Reverse gyrase was initially discovered in *Sulfolobus acidocaldarius,* a thermoacidophilic sulphur metabolizing archaebacterium by Kikuchi and Asai, 1984. The majority of work on reverse gyrase since this time has been on this organism. Slesarev, 1988 and his co-worker have purified and extensively characterized reverse gyrase from *Desulfurococcus amylolyticus* which belongs to the same group of extremely thermophilic sulphur metabolizers

but to a different order within this group. It appears that reverse gyrase is restricted to the thermophilic sulphur metabolizing group of archaebacteria as determined by *in vitro* activity on crude cell lysates. Thermophilic methanogens and eubacteria and all mesophilic organisms screened had no reverse gyrase activity (Collin *et al.*, 1988). Anti-reverse gyrase antibodies have been used in an attempt to determine cross-reactivity with other organisms. Extracts of *E. coli*, *Thermus thermophilus*, *Halobacterium halobium*, yeast, and *Drosophilia melanogaster* were all tested and no specific cross-reactivity was observed (Kikuchi *et al.*, 1986).

1.3.5 The biological roles of reverse gyrase

One of the most likely biological functions of reverse gyrase is the stabilization of DNA by positive supercoiling (Kikuchi and Asai, 1984). The fact that single-stranded DNA is the preferred substrate for reverse gyrase catalysis suggests a biological role for this enzyme. Melted regions of chromosomal DNA would be preferentially catalysed by reverse gyrase and the positive supercoiling which results would bring about the renaturation of the melted region. Functions such as transcription and replication which cause strand separation of the double helix would therefore be followed by reverse gyrase activity to enhance renaturation of melted regions (Shibata *et al.*, 1987). This type of activity would be especially useful in thermophilic environments where strand renaturation might occur with difficulty.

A second topoisomerase has been found in *Sulfolobus*. This type II enzyme is likely to be a DNA gyrase (Nakasu and Kikuchi, 1985; Nadal *et al.*, 1986; Sioud *et al.*, 1988). This suggests that another role for reverse gyrase is to act as an antagonist to this enzyme leading to an equilibrium in the supercoiled state of the chromosomal DNA. Slesarev and Kozyavkin, 1989 have suggested that both reverse gyrase and eubacterial type I topoisomerases serve a similar purpose which is to control DNA duplex stability near its minimum level. This control is brought about by a single-stranded substrate specificity where the enzymes act on melted regions of the DNA and dissociate from the DNA when the helix is stable. This action combined with an opposing DNA gyrase action would confine chromosomal supercoiling to a narrow range characterized by low stability of the double helix. The level of supercoiling needed to achieve a marginally stable duplex at moderate temperatures would be at an appropriate negative

superhelicity while at extremely high temperatures positive superhelicity is required (Slesarev and Kozyavkin, 1989).

The advantage of regulating intracellular supercoiling where the helix is of low stability with a high likelihood of fluctuational openings but without melting of DNA, is that DNA protein interactions are favoured and the detrimental effects of regions of melted DNA are avoided.

Another possible role for reverse gyrase is in the area of gene regulation and expression. It has been suggested that reverse gyrase, by positive supercoiling of parts of the genome, could transform cruciform structures or segments of Z-DNA back into regular B-DNA (Kikuchi and Asai, 1984). The transition of such structures in chromosomes may be important as initiation signals for DNA replication, recombination, and gene expression (Nordeim and Rich, 1983; Kohwi-Shigematsu *et al.*, 1983).

1.4 Genome organisation and supercoiling in archaebacteria and other organisms

This section provides a brief summary on genome organisation and intracellular supercoiling in eubacteria, eukaryotes, and archaebacteria. This is of interest to the thesis topic for two main reasons: Firstly, supercoiling of DNA, either as free plectonemic supercoiling or as constrained supercoils wrapped around proteins, is an essential part of genome organisation in all organisms thus far studied. It seems likely that both the free level of supercoiling in the cell and protein binding have an effect on the thermostability of DNA (Slesarev and Kozyavkin,1990; Reddy and Suryanarayana, 1988). Second, genome organisation between eubacteria and eukaryotes appears to be markedly different (Schmid, 1988) and a comparison with the known facts about the archaebacterial genome may reveal important relationships with other kingdoms.

1.4.1 Genome organisation in eubacteria

Eubacteria contain about 4×10^6 base pairs of genomic DNA with a total length of 1.4mm. This is packaged in a cell whose typical length is about 2um. It follows that the DNA must be folded at least 700 times in order to fit within the structure of the cell (Woolley, 1986). In logarithmicaly growing cells, which can contain up to four chromosomes, the concentration of DNA in the cell can reach 17 mg/ml^{-1} (Woolley, 1986). It has been estimated that 70% of the *E.*

coli genome (or about 2800 genes) can encode protein products while 10% of the DNA is likely to be intergenic and perhaps reserved for a structural role in the genome (Schmid, 1988). For protein encoding portions of the genome to be transcribed it follows that the physical organisation of the DNA must accommodate the need for recognition by proteins and enzymes involved in transcription and replication.

These points on chromosomal DNA show that DNA must be condensed to a large extent within the cell, however the DNA must also be in a sufficiently exposed state to allow the diffusion and recognition of DNA binding proteins to occur. A model accommodating these features is described in the following paragraphs:

In bacteria there appear to be two distinct levels of DNA condensation; the organisation of independently supercoiled domains of DNA averaging 100kbp in size and the organisation of shorter segments of DNA (60-120bp) with abundant DNA-binding proteins (Schmid, 1988):

(a) Higher level organisation of genomic DNA into individual domains (100kbp per domain). The eubacterial genome is normally circular and in *E. coli* is organised into about 43 ± 10 independently supercoiled domains (Sinden and Pettijohn, 1981). DNA supercoiling can be relaxed in one domain while not affecting the supercoiling in other domains (Pettijohn, 1988). This opens up the possibility for the regulation of groups of genes that form a chromosomal domain. At this level the bacterial chromosome is condensed by two factors: 1. The folding of the chromosome into domains. 2. The supercoiling of DNA within domains see figure 1.4.1.

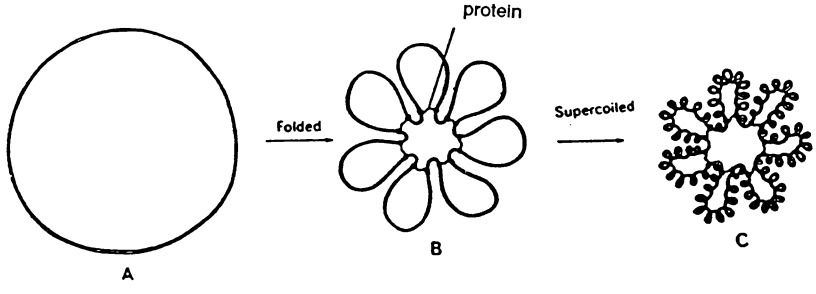


Figure 1.4.1 A model for the condensation of the bacterial chromosome (modified from a diagram in Pettijohn and Hecht, 1974). (A) The circular, unfolded chromosome. The diameter of the circle is 350um. (B) The chromosome folded into domains by DNA-protein interactions at the base of the domains. Seven domains are shown for simplicity. The diameter, assuming 40 equal domains, is about 30um. (C) The chromosome folded into domains which are supercoiled. The diameter of the chromosome is about 2um.

It is likely that supercoiled domains are stabilised by DNA-protein interactions (Sloof *et al.*, 1983). These proteins are considered to be membrane associated (Schmid, 1988) and a likely hypothesis for eubacterial domain organisation is that chromosomal DNA is organised into domains by DNA-protein-membrane interactions. Although no direct evidence has shown that membrane attachment has a role in domain formation or stability it is implicated by many indirect observations (Ogden and Schaechter, 1986). It is likely that DNA gyrase is one of the proteins associated with formation of domains (Snyder and Drlica, 1979) as DNA gyrase is distributed along genomic DNA at 100kbp intervals which corresponds to 1 gyrase per domain (Drlica, 1984). DNA gyrase could act at the base of a DNA domain, perhaps not only to ensure domain integrity, but also to control its level of superhelicity. It is interesting that this function has also been suggested for the eukaryotic type II topoisomerase (see section 1.4.3).

(b) Lower level organisation of DNA into nucleosome-like structures (120bp per structure). Eubacterial DNA appears to be associated with histone-like proteins in a manner which is visually reminiscent of eukaryotic nucleosome packaging (Griffith, 1976; Pettijohn, 1982). DNAase digestion of total bacterial nucleoprotein has revealed that 120bp DNA fragments are protected by abundant, small DNA-binding proteins (Varshavsky *et al.*, 1977). Five different eubacterial proteins have biochemical properties which resemble eukaryotic histones. They are small, normally basic proteins, found in high intracellular concentrations. For example the four main histone-like proteins found in *E. coli* are H (~120,000 monomers per cell), HU (~60,000 monomers per cell), H1 and IHF (14,000 to 20,000 copies per cell) (Pettijohn, 1988).

The *in vivo* functions of these proteins have not been elucidated and whether they interact with each other to form a multi-protein complex, act singly to condense DNA, or have a role in the cell unrelated to DNA condensation is not known. The problem in assigning a function to histone-like proteins arises as bacterial chromatin appears to be extremely labile and so far no isolation procedure has been developed which can extract and chracterize the putative histone-like proteins from bacterial chromatin (Drlica and Rouvière-Yaniv, 1987). It has also been postulated that the polyvalent cations spermidine and putrescine, which exist in 5-10mM concentrations in bacterial cells, may play a role in the condensation of bacterial DNA (Schmid, 1988).

The histone-like protein HU is the most intensively studied of these proteins. Purified *E. coli* HU protein binds 58bp of DNA per HU tetramer and the DNA is wrapped around HU in a left-handed toroid. This wrapping is in the same direction as the wrapping of eukaryotic DNA in the nucleosome and both result in restrained negative supercoiling of DNA around the protein complex (Rouvière-Yaniv *et al.*, 1979). The compensating positive supercoiling of free DNA induced when DNA is bound in a negative supercoil around HU is presumably removed by the action of DNA gyrase. It has been calculated that the cellular levels of HU protein should be enough to cover about 20% of the DNA found in the bacterial cell (Schmid, 1988) thus the level of restrained negative supercoiling in the cell could be significant.

A model for the wrapping of DNA by HU tetramers has been developed by Drlica and Rouvière-Yaniv, 1987 (figure 1.4.2).

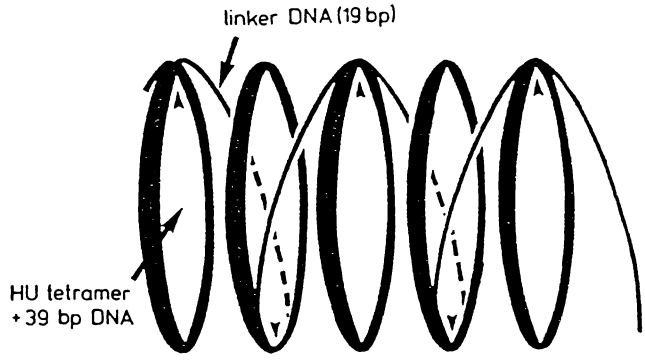


Figure 1.4.2 Model for DNA wrapping by HU (reproduced from Drlica and Rouvière-Yaniv, 1987). DNA is wrapped around tetramers of HU such that 290bp makes 7.5 turns around five tetramers of HU. Each turn consists of 38 to 39bp, and each linker is about 19bp long. Thus adjacent tetramers are inverted relative to each other.

The model is based on the following considerations. For each linking change of -1, HU binds to about 290bp of DNA (Rouvière-Yaniv *et al.*, 1979). Sites hypersensitive to nucleases occur at 58bp intervals (Broyles and Pettijohn, 1986), so that some structure occurs five times for each change in linking number of 1 (290/58 = 5). The repeating structure is probably a tetramer of HU since 20 monomers bind per 290bp of DNA (Mende *et al.*, 1978). Nuclease-sensitive sites also occur every 8.5bp (Broyles and Pettijohn, 1986). If this reflects a change in the helical pitch of DNA to 8.5bp per turn, there would be 2bp fewer per turn then found with DNA in solution. Over the 290bp of wrapped DNA, there would be 6.5 turns of additional twist. This means that if the nucleosome-like particle has a linking change of -1 then the DNA would describe a path having 7.5 helical turns (1.5 turns per tetramer of HU).

In this model, HU alone is sufficient to produce a nucleosome-like structure and the interactions of other histone-like proteins are not considered.

The interaction of *E. coli* H1 with DNA has also been studied. It appears that this histone-like protein can significantly compact DNA *in vitro* though the writhing of the DNA molecule

remains essentially unchanged (Rimsky and Spassky, 1986). It was concluded that H1 could participate in the efficient packaging of DNA in the bacterial nucleoid.

1.4.2. *In vivo* supercoiling in eubacteria

The *in vivo* supercoiling of eubacteria can be divided into two components:

Firstly the restrained supercoiling of DNA around proteins, for example HU. This results in negative superhelical wrapping of DNA on the protein complex with a concomitant positive supercoiling outside the DNA-protein structure which can be relaxed by a topoisomerase. Negative supercoiling which is constrained by protein interactions, i.e. under no torsional tension, contributes about 60% of the total negative supercoiling in a eubacterial cell (Bliska and Cozzarelli, 1987; Pettijohn and Pfenninger, 1980).

The second component of supercoiling is the unrestrained supercoiling of DNA which results in the plectonemic writhing of DNA to produce a structure which is under torsional tension (Bliska and Cozzarelli, 1987). This component of supercoiling, called the effective superhelical density, accounts for about 40% of the total negative supercoiling in a eubacterial cell (Pettijohn, 1988). The average effective superhelical density is the result of the steady state action of DNA gyrase and other topoisomerases on DNA. Other biological processes also affect supercoiling, e.g. transcription and replication induce supercoiling which can however be removed by topoisomerases.

The effective superhelical density of DNA *in vivo* can be estimated by a number of different approaches. Some of the first approaches in determining the superhelical density of the bacterial genome were to isolate the entire chromosome as a nucleoid and run these on sucrose gradients with varying concentrations of ethidium bromide (Worcel and Burgi, 1972). The results of these experiments from *E. coli*, *Salmonella typhimurium*, *Bacillus subtilis* and *B. licheniformis* gave superhelix densities of -0.129 to -0.17 for the isolated bacterial nucleoid (Sloof *et al.*, 1983; Dworsky, 1976). These values are not thought to represent the effective superhelical density of DNA *in vivo* as many DNA-binding proteins are removed from the nucleoid upon its isolation which would increase its apparent negative superhelicity (Drlica and Rouvière-Yaniv, 1987) and futhermore, ethidium bromide-protein association would also result in an overestimation of the

superhelix density (Sloof *et al.*, 1983).

One simple method used to determine the effective superhelical density is to determine the superhelical density of purified cccDNA and use 40% of this value. Thus purified cccDNA isolated from eubacteria have a typical superhelical density of -0.06 (Giaever *et al.*, 1988) If 60% of this is in a restrained form then this leaves an effective superhelical density of -0.024 (40% of the total).

A more direct approach, using the supercoiling dependent binding of psoralen to DNA, has been used (Sinden *et al.*, 1980). The rate of binding by trimethylpsoralen is linearly related to the superhelical density of DNA and this relationship was determined *in vitro*. This was then used to calibrate the effective superhelical density *in vivo*. In eubacteria it was discovered that DNA in the form of torsionally strained negative supercoils was present at an apparent superhelix density of -0.05 (Sinden *et al.*, 1980). However this value may be condidered low as the effect of DNA bound proteins on the rate of psoralen binding was unknown (Bliska and Cozzarelli, 1987).

Another approach compares promoter expression on plasmid pAS21 *in vitro* and *in vivo* (Borowiec and Gralla, 1987). The superhelical density that provides the best match between the levels of expression *in vivo* and *in vitro* was found to be $\sigma = -0.48 \pm 0.01$.

A different approach which depends upon the formation of alternate DNA structures has been investigated by several groups. This approach has given an effective *in vivo* superhelical density in eubacteria of about -0.025 and this figure is generally regarded as the most reliable estimate of effective supercoiling *in vivo* available. Zacharias *et al.*, 1988 have used this technique by inserting lengths of DNA into plasmids which are capable of forming Z-DNA. The effective superhelical density needed to generate B to Z transitions was examined *in vitro* and compared with the generation of Z-DNA from the same plasmids *in vivo*. The results from this experiment gave an effective superhelical density of -0.025. A similar experiment, which used a different technique to detect Z-DNA, suggested an effective *in vivo* superhelical density of -0.035 (Sinden and Kochel, 1987) while an experiment which used cruciform formation as an indicator of effective superhelix density gave $\sigma = -0.024$ (Lilley, 1986). A recent study which utilises OsO_4 to positively detect Z-DNA *in vivo* has shown that short naturally occuring sequences of DNA found in *E. coli* can undergo the B to Z transition (Rahmouni and Wells, 1989). It was found that the

localized superhelical density of these segments cloned into pBR322 could be as high as -0.038 and as low as -0.021 depending on the position of the DNA segment in relation to transcribed regions of the plasmid. Thus the transient levels of supercoiling in a cell in regions of transcription may vary markedly from the steady state effective superhelicity of the genome (Wu *et al.*, 1988). Transient supercoiling produced by transcription may affect DNA structure; for example the waves of negative supercoiling generated behind the transcription machinary can be sufficient to transiently induce non B-DNA structural transitions (Tsao *et al.*, 1989).

One problem with these approaches is that the reaction conditions *in vitro* do not closely resemble the physiological state *in vivo* and the reaction may vary according to specific ion concentration, temperature, protein concentration, DNA structure, and the presence of binding proteins or topoisomerases (Bliska and Cozzarelli, 1987). Recently Kozyavkin *et al.*, 1989 have disputed the generally held theory that the energy of supercoiling is quadratically related to a change in superhelix density. They have suggested that the elastic energy of DNA depends not only on superhelix density but also on the surrounding ions and possibly proteins. They have concluded that this would affect the *in vitro* calibration estimates used to determine the effective superhelical density in most of the above experiments. For example Kozyavkin *et al.*, 1989 looked at the effect of cations on cruciform formation. Under conditions containing only monovalent cations cruciform formation was favoured (these conditions are similar to those used in most *in vitro* estimates of DNA structural transitions) whereas under conditions approximating the *in vivo* environment in *E. coli* (10mM $MgCl_2$ and 6mM spermidine) cruciform formation did not occur. It would therefore seem that it is necessary for experiments which use an approach involving *in vitro* calibration to be reappraised before any final estimate of an effective *in vivo* superhelical density is concluded. Furthermore it is possible that *in vivo* supercoiling will vary according to environmental circumstances and the state of the cell. For example a change in the osmotic environment is known to effect *in vivo* supercoiling (Higgins *et al.*, 1988) as is the production of spores from *Bacillus subtilus* (Nicholson and Setlow, 1990) and a change of oxygen tension (Dorman *et al.*, 1988).

1.4.3. Genome organisation in eukaryotes

The organisation of DNA within eukaryotes has been reviewed on many occasions (see for example Butler, 1983; McGhee and Felsenfeld, 1980). The bulk of the DNA in eukaryotic cells exists in the form of a compact nucleoprotein structure termed chromatin. The high overall packing ratio of this genetic material suggests that DNA is not directly packaged into the highest level of chromatin and that there are a number of levels of chromatin organisation within the cell.

At the lowest level there is the elementary unit of chromatin termed the nucleosome. This compacts DNA by about 6x. At the medium level of organisation the nucleosomes are coiled into a helical array to constitute what is called the 30nm fibre. This brings the packing ratio to 40x. At the highest level the 30nm fibre is further compacted into loops each of which is thought to constitute a separate superhelical domain. This gives an overall packaging ratio of 1000-10000 in eukaryotic cells (Lewin, 1987).

Eukaryotic chromatin contains roughly twice as much protein as DNA. This protein component can be divided into two groups; the histone proteins of which there are five classes (H1, H2A, H2B, H3, H4) and which account for about the same mass as the DNA, and the non-histone proteins which are a diverse group including all other proteins found in chromatin. The non-histone proteins include proteins with functions concerned with gene expression and with higher order structure. Prominent proteins in this group include RNA polymerase and DNA topoisomerases I and II (Lewin, 1987; Gilmour *et al.*, 1986).

(a) Low level organisation (the eukaryotic nucleosome). The nucleosome, which is the basic unit of all chromatin, contains roughly 200bp of DNA associated with a histone octamer. This octamer consists of two copies each of the histones H2A, H2B, H3, and H4 in a configuration where a tetramer of $(H3.H4)_2$ lies at the centre of the octamer with a (H2A.H2B) dimer lying on either side (Ollis and White, 1987). This histone complex has a disc-like structure 57Å thick and 110Å in diameter. DNA is bound on the outside of the complex and wraps around the histone complex 1.8 times in a shallow helical path (figure 1.4.3). The DNA helix is severely deformed by its incorporation into nucleosomes and is wrapped around the histone complex in a series of kinks rather than by a smooth curve (Ollis and White, 1987).

The binding of histone octamers to DNA results in the restrained negative superhelical wrapping of DNA on the histone complex that is equivalent to a change in writhing number of about 1.25 per nucleosome (Gellert, 1981a). The discrepency between the expected change in writhe of -1.8 (due to DNA wrapping around the histone complex in 1.8 turns) and the actual writhe of -1.25 is best explained by a twist component of -0.55 which is introduced solely by virtue of the three-dimensional pathway of DNA on the surface of the nucleosome (White and Bauer, 1988; Morse and Simpson, 1988) rather than by an alteration of local twist from 10.6 to 10.0 on the nucleosome (Lewin, 1987) which has been the generally accepted explanation.

In their natural state, nucleosomes are likely to be closely packed with DNA passing directly from one to the next (figure 1.4.4).

(b) Mid level organisation (The 30nm fibre). At this level of organisation the closely packed nucleosomes are further arranged so that they form a solenoidal structure (figure 1.4.5). This solenoid has about six nucleosomes for every turn, which corresponds to a packing ratio of 40 (that is, each um along the axis of the fibre contains 40um of DNA) (Lewin, 1987). The presence of histone H1 is necessary for the formation of the solenoid and it is apparently responsible for the folding of nucleosomes into this helical structure (Butler, 1983).

(c) High level organisation (Loops of 30nm fibre). At this highest level of organisation it appears that the 30nm fibre, made up from a solenoidal array of nucleosomes, is arranged into a series of radial loops which emanate from near the centre of the arms of the chromosome (Marsden and Laemmli, 1979; Adolph, 1981). These loops or domains of DNA are independently supercoiled and so must be restrained at the base of the loop by some structure (Benyajati and Worcel, 1976). These loops of DNA are about 85,000 to 100,000bp long (Benyajati and Worcel, 1976; Cook and Brazell, 1975) which is similar to the estimated length of the domains of DNA found in eubacterial chromosomes.

The loops of 30nm fibre are arranged so that the base of the loop is 'tied' to the chromosome scaffold. This scaffold is made up of non-histone proteins which interact together to give a regular, and possibly helical, array along the axis of the chromosome (Butler, 1983). One of these scaffold proteins is topoisomerase II (Earnshaw *et al.*, 1985). Topoisomerase II is associated with the base of DNA loops and may even be the protein responsible for attaching the

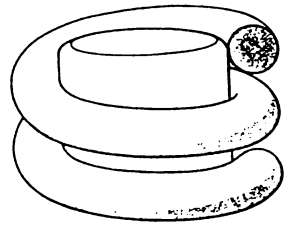


Figure 1.4.3 A model showing the wrapping of DNA around the eukaryotic histone octomer. The DNA is believed to wrap around the DNA around the octomer to form a left handed helix of about 200 base pairs (reproduced from Lewin,1987).

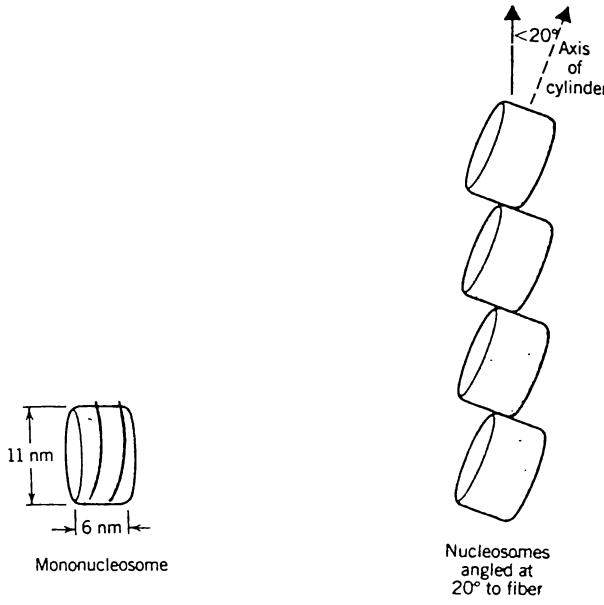


Figure 1.4.4 A model showing the packing of nucleosomes togather at an angle of 20° to form what is known as the 10nm fibre (reproduced from Lewin, 1987).

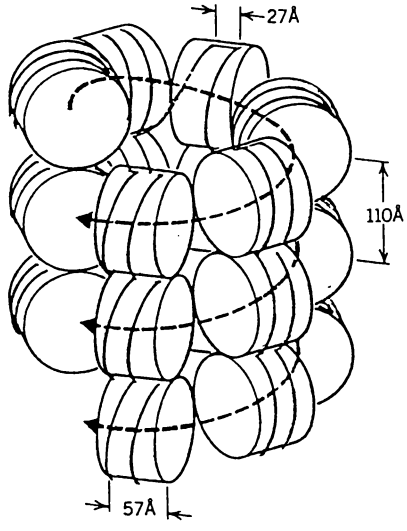


Figure 1.4.5 A model showing the packing of nucleosomes into a solenoidal structure known as the 30nm fiber (reproduced from Lewin, 1987).

DNA loop to the chromosome scaffold (Pommier *et al.*, 1989). If this is the case then it is ideally placed to regulate the topological state and perhaps transcriptional activity of such a DNA domain (North, 1985). It has been suggested that topoisomerase II plays an essential role in the higher order folding of DNA which is necessary for the compaction observed in chromosomes (Roca and Mezquita, 1989) and if this is the case it would confirm that this enzyme is an integral part of the chromosome scaffold in eukaryotes.

The above description for DNA folding in eukaryotic chromatin is concerned with the structure of the bulk of the cellular chromatin which in a typical eukaryotic cell is not transcriptionally active. The situation in transcriptionally active chromatin is different and while the DNA domains remain intact the mid level structure is disrupted during transcription. Most of the DNA seems to be bound up in nucleosomes during transcription except perhaps for DNA involved in very high levels of transcription such as genes encoding rRNA (Butler, 1983). It appears that nucleosomal DNA uncoils from the histone octamer without releasing it completely as RNA polymerase passes during transcription (Alberts *et al.*, 1989).

A major difference between eukaryotes and eubacteria is that virtually all bacterial genes are available for transcription while the majority of the genes in higher organisms are not. One attractive idea is that bacterial chromatin is similar to the minor class of eukaryotic chromatin that is transcriptionally active.

The organisation of the bulk of eukaryotic chromatin differs from that found in eubacterial chromatin in several important respects. (1) Eukaryotic nucleosomes are comprised of 4 histone types while the nucleosomes themselves are organised into a repeating structure. This is not true of DNA bound by the eubacterial HU protein. (2) Eukaryotic chromatin has an intermediate level of DNA compaction that arises from binding of the histone H1 and coiling of nucleosome into a solenoid. No analog of this compaction has been found in eubacteria. (3) The bulk of eukaryotic DNA seems unstrained, while the bulk of bacterial DNA seems torsionally strained (Schmid, 1988).

1.4.4. *In vivo* supercoiling in eukaryotes

The superhelical density of DNA isolated from eukaryotic sources is similar to that isolated from eubacterial organisms and shows the same differentiation of superhelical density between chromosomal and cccDNA. For example isolated eukaryotic chromosomes loaded onto ethidium bromide/sucrose density gradients display a typical superhelical density of around -0.13 (found for *Drosophila melanogaster*, Hela cells, and *Xenopus laevis*) (Sloof *et al.*, 1983). This is in contrast to eukaryotic viral, plasmid or mitochondrial cccDNA which have values typically around -0.06 (Giaever *et al.*, 1988; Shen and Hu, 1985). This difference can be reconciled, similarly to eubacterial counterparts, by differences in DNA packaging and ethidium bromide-protein association in eukaryotic chromosomes which would result in an overestimation of the superhelix densities of the chromosomes (Sloof *et al.*, 1983).

In constrast to eubacterial DNA, the supercoiling of eukaryotic DNA can be entirely attributed to the formation of nucleosomes so that all of the supercoiling in the cell is in a restrained form. Unrestrained DNA is not detected although the experiments used to determine this result could not preclude the existence of torsionally strained DNA in a small fraction of the chromosome (Sinden *et al.*, 1980). Entirely restrained supercoiling in eukaryotic chromosomes is further implicated by the change of linking number found in SV40 DNA isolated from infected cells. This equated with the change expected from the binding of DNA to the nucleosomal structure alone (Germond *et al.*, 1975). Furthermore a change in supercoiling is not noticed in yeast cells when DNA topoisomerase I or II is inactivated implying that DNA is torsionally unstressed in these cells (Giaever *et al.*, 1988; Wang, 1987).

Although the bulk of DNA in eukaryotes is thought to be in a restrained state this does not rule out the possibility that a small proportion of the chromosome could contain torsionally stressed DNA and this has in fact been implicated for those regions of DNA undergoing transcription. Three lines of evidence suggest that this may be the case. Firstly, portions of chromatin in transcriptionally active states are hypersensitive to reagents which react preferentially to negatively supercoiled DNA (Kohwi-Shigematsu *et al.*, 1983; Weintraub and Groudine, 1976). Second, the transcription of many eukaryotic genes is enhanced if they are negatively supercoiled (Han *et al.*, 1985; Luchnik *et al.*, 1982; Ryoji and Worcel, 1984, and

Villepontea *et al.*, 1984). For example a 500-1000 fold increase in the template activity of a circular negatively supercoiled plasmid injected into toad oocytes occurs, as compared with the same plasmid in the linear state (Harland *et al.*, 1983). The results from the experiments of Harland *et al.*, 1983; Luchnik *et al.*, 1982, and Ryoji and Worcel, 1984 suggest that a significant proportion of minichromosomes *in vivo* exist in a torsionally strained state and that this strained subpopulation is transcriptionally active (Shen and Hu, 1986). Third, yeast strains which lack DNA topoisomerase I, contain actively transcribed plasmids which are highly negatively supercoiled (Brill and Sternglanz, 1988). This suggests that in normal yeast cells negative supercoiling arises during transcription and that topoisomerase I is responsible for removing this excess supercoiling.

The production of torsionally strained DNA in or near transcribed regions has been explained by the unbinding of nucleosomes, a DNA gyrase like activity incorporating DNA topoisomerase II, and the effect of transcription on DNA supercoiling. Brahms *et al.*, 1985 have suggested that the unfolding of DNA wound around the nucleosomal core could generate torsional stress particularly in the transcription of ribosomal genes where histones are totally dislodged. However this explanation is not sufficient to account for torsional strain in the majority of transcribed regions which contain nucleosomes. A second, and widely published, explanation of regions of torsional stress is the negative supercoiling of DNA by the eukaryotic DNA topoisomerase II, possibly in conjunction with a second protein (Gliken *et al.*, 1984; Hirose and Suzuki, 1988; Kmiec and Worcel, 1985). This was postulated by studies using the topoisomerase II inhibitor novobiocin which implied that topoisomerase II was required to maintain the torsionally stressed character of active genes in chick erythroid cells (Villeponteau, *et al.*, 1984), for the induction of the hsp70 genes in *Drosophila* (Han *et al.*, 1985), and in 5S gene activation in *Xenopus* (Ryoji and Worcel, 1984). Furthermore extracts of eukaryotic cells and partially purified fractions have been reported to negatively supercoil cccDNA (Glikin *et al.*, 1984; Hirose and Suzuki, 1988) and DNA topoisomerase II was implicated to be one of the supercoiling proteins. However, as Giaever and Wang, 1988 observed, these *in vitro* systems were not sufficiently purified to unequivocally establish the mechanism of the observed DNA linking number reduction. Furthermore the eukaryotic DNA topoisomerase II does not supercoil DNA *in vitro*

(Vosberg, 1985; Wang, 1985) or *in vivo* (Giaever *et al.*, 1988; Saavedra and Huberman, 1986). The third, and most probable, explanation is that transcription in conjunction with topoisomerase activity results in regions of negatively supercoiled torsionally strained DNA. As proposed by the twin supercoiled domain model (See section 1.5) the transcription process generates local positively and negatively supercoiled regions on either side of the transcription complex. If the positively and negatively supercoiled loops are relaxed at similar rates by the topoisomerases they cancel each other and do not contribute to the average value. However if the supercoiled regions are relaxed differentially then the accumulation of supercoils of one sense or the other can occur (Liu and Wang, 1987). Both eukaryotic DNA topoisomerases can relax positively and negatively supercoiled domains *in vivo* (Giaever *et al.*, 1988) however the rates of relaxation for the DNA substrate may not only differ but depend upon the sign of supercoiling. Thus it appears that DNA topoisomerase I can relax negative supercoils more efficiently than DNA topoisomerase II (Brill and Sternglanz, 1988). Furthermore, DNA topoisomerase II preferentially relaxes positive supercoils as inhibition of this enzyme leads to an increase in linking number (Villeponteau *et al.*, 1984). One theory that could therefore explain the observed negative supercoiling in transcribed regions is as follows:

1. Transcription produces a superhelical domain in front of and behind the transcription complex. The domain in front of the complex is more positively supercoiled while the domain behind the complex is more negatively supercoiled.
2. DNA topisomerase I acts to preferentially remove negative supercoils.
3. DNA topoisomerase II acts to preferentially remove positive supercoils.
4. If DNA topoisomerase II acts at a faster rate, then the general region around the transcription complex will exhibit a nett negative superhelicity.

1.4.5 Genome organisation in archaebacteria

Little is known about the genomic structure of archaebacteria as compared to eubacteria or eukaryotes. Archaebacteria, like eubacteria, contain genomic DNA which is a single circular DNA molecule (though halobacteria contain extra 'satellite' DNA) and ranges in size from 8.4 x 10^8Da for *Thermoplasma acidophilum* (Searcy and Doyle, 1975) to 2.3 x 10^9Da for

Halobacterium halobium and *Halobacterium morrhuae* (Moore and McCarthy, 1969). This range of values is reasonably comparable to that found in the eubacterial kingdom although some eubacterial members contain chromosomes which are significantly larger than 2.3 x 10^9Da (Herdman, 1985).

Species of *Halobacterium* and *Halococcus* contain both a 'chromosomal' DNA component and a 'satellite' DNA component. This latter fraction comprises 11 to 36% of the total DNA (Doolittle, 1985).

Most of the information about the structure of archaebacterial chromatin has come from studies of DNA binding proteins isolated from these organisms. Histone-like and DNA-associated proteins have been identified and characterised from species in all phylogenetic branches of the archaebacterial kingdom. The most thoroughly characterised histone-like protein, HTa from *Thermoplasma acidophilum*, displays an intriguing amino acid sequence similarity to both the eubacterial histone-like protein HU and to eukaryotic histones (DeLange *et al.*, 1981b). Thus it has been suggested that the *Themoplasma* protein HTa is the link that can relate eukaryotic histones to the bacterial histone-like proteins (Searcy, 1986). However the properties of HTa resemble those described for eukaryotic histones rather than eubacterial histone-like proteins, for example: (1) HTa remains attached to DNA during isolation of the chromosome (Searcy, 1975); (2) it binds tightly to DNA throughout the entire range of intracellular ionic strengths but can be dissociated by salt concentrations about ten times greater than physiological (DeLange *et al.*, 1981a); (3) it condenses DNA into small uniform globular particles that resemble eukaryotic nucleosomes (Searcy and Stein, 1980); and (4) it is very effective in protecting DNA from thermal denaturation and nuclease digestion (Stein and Searcy, 1978). HTa binding increases the temperature at which dsDNA molecules denature by as much as 40°C (Stein and Searcy, 1978).

Histone-like proteins can be isolated from *Sulfolobus acidocaldarius*. One such protein isolated by Green *et al.*, 1983 displayed properties which were intermediate between those of the *E. coli* HU protein and *T. acidophilum* protein HTa. Four histone-like proteins were isolated from *S. acidocaldarius* by Reddy and Suryanarayana, 1988. Three of these proteins increased the thermal stability of double-stranded DNA by 25°C. These workers concluded that the three histone-like proteins were similar to eukaryotic histones as evidenced by: (1) association with

intracellular DNA; (2) amino acid composition; and (3) protection of DNA against thermal denaturation.

Similar proteins have been found in other archaebacteria including *Thermococcus celer* (Dijk and Reinhardt, 1986) and *Methanothermus fervidus* (Brown *et al.*, 1989; Sandman *et al.*, 1990).

The above histone-like proteins and the nucleosome-like structures that they form (at least for HTa) are as Searcy, 1982 states "suggestively eukaryotic". Whether this is due to evolutionary relatedness or convergence is not clear and the extent to which the archaebacterial genome resembles the eukaryotic or eubacterial genomes is, at the present time, unknown.

1.4.6 *In vivo* supercoiling in archaebacteria

Within the archaebacterial kingdom there may well be significant differences in DNA topology between the thermophilic sulphur metabolizing archaebacteria and other archaebacterial groups. This difference is postulated due to the existence of the positively supercoiling reverse gyrase enzyme in the first group and its presumed absence in the latter groups (Collin *et al.*, 1988). Such a difference is a significant feature and bears out the deep dichotomy which is evident between the sulphur metabolizing archaebacteria and the other archaebacterial groups (methanobacteria, halobacteria, and *Thermoplasma*) (Brown *et al.*, 1989; Lake, 1988; Zillig *et al.*, 1988). Halobacteria appear to possess negatively supercoiled DNA as evidenced by the isolation of a negatively supercoiled plasmid from *Halobacterium* GRB (Sioud *et al.*, 1988) and *Halobacterium* strain 5 (Simon, 1978). Whether this supercoiling is in a restrained or unrestrained form *in vivo* was not determined. Presumably the closed circular plasmids which have been isolated from other species of halobacteria and methanobacteria (Brown *et al.*, 1989) were negatively supercoiled though this was not explicitly stated.

Although it has been postulated that the genome of sulphur metabolizing archaebacteria may possess positively supercoiled DNA (Nadal *et al.*, 1986), or at least regions of positively supercoiled DNA, through the action of reverse gyrase, the only direct indication that this is so has come from studies on DNA from a virus-like particle isolated from *Sulfolobus acidocaldarius* (Nadal *et al.*, 1986). This covalently closed circular DNA was found to be, on the whole, positively

supercoiled. Viral DNA isolated from the virus particle itself was found to be fully positively supercoiled whereas viral DNA isolated from the bacterium contained a wide spectrum of positive topoisomers and a small proportion of negative topoisomers. Nadal *et al.*, 1986 suggest that the discrepancy between these two forms is due to the positively supercoiled viral DNA becoming negatively supercoiled during transcription and replication within the cell after which it is condensed into a highly positive supercoiled form ready for encapsidation. The existence of negatively supercoiled DNA and positive supercoils of moderate superhelicity suggest that another topoisomerase, which acts as an antagonist of reverse gyrase, is present. Such a topoisomerase has been isolated from a *Sulfolobus* strain (Nakasu and Kikuchi, 1985).

Taking into consideration the isolation of positively supercoiled DNA from a sulphur metabolizing archaebacteria and the preponderence of positively supercoiling activity in crude extracts from these organisms it seems possible that the genomes of these bacteria are positively supercoiled. The extent of this supercoiling is uncertain and no doubt reflects the actions of reverse gyrase and an opposing topoisomerase, the degree of DNA-protein interaction and the effect of restrained supercoiling around histone-like proteins, and the effect of transcription. It seems certain that if the genome is predominantly positively supercoiled that negatively supercoiled regions or domains could exist perhaps in regions where replication or transcription were occuring.

1.5 The twin-supercoiled-domain model and regulation of supercoiling

1.5.1 Regulation of supercoiling

The regulation of supercoiling in bacteria is important as superhelical density affects transcription where promoters and some repressor-operator interactions are strongly affected (Dimri and Das, 1988; Rudd and Menzel, 1987; Whitson *et al.*, 1987), so the expression of particular genes at an inappropriate level or time could be detrimental. Furthermore, other vital functions, such as replication, recombination, and transposition are also affected by supercoiling levels (Baker, *et al.*, 1986; Funnell *et al.*, 1986; Isberg and Syvanen, 1982; Rich *et al.*, 1986).

The intracellular state of DNA supercoiling in eubacteria is controlled by two main

opposing topoisomerases, DNA topoisomerase I which relaxes negative supercoils and DNA gyrase which relaxes positive supercoils and introduces negative supercoils. In *E. coli*, and presumably other eubacteria, these two enzymes appear to be homeostatically regulated, a decrease in the degree of negative supercoiling elevates the transcription of the *gyr*A and *gyr*B genes, encoding the two subunits of DNA gyrase, and reduces the transcription of the *top*A gene, encoding DNA topoisomerase I. An increase in the degree of negative supercoiling has the opposite effects on the expression of these genes (Gellert *et al.*, 1982; Tse-Dinh, 1985; Tse-Dinh and Beran, 1988).

*E. coli top*A mutants are not viable unless they acquire compensatory mutations, some of which reduce the level of DNA gyrase (Pruss *et al.*, 1982; Raji *et al.*, 1985). The suppression of the lethal phenotype of *top*A mutations by compensatory mutations in the *gyr*A and *gyr*B genes supports the view that an appropriate level of intracellular supercoiling is essential and is maintained primarily by a dynamic balance between the actions of these two opposing topoisomerases. The regulation of DNA supercoiling occurs both at the transcriptional level and at the level of topoisomerase-DNA interactions where the activities of the opposing topoisomerases display an inverse dependence on the topological state of the DNA substrate. Thus DNA topoisomerase I activity declines as the DNA substrate becomes increasingly more relaxed (Wang, 1971) whereas DNA gyrase's highest affinity is for relaxed DNA (Morrison *et al.*, 1980).

This simple model for the regulation of supercoiling in cells leaves a number of observations unexplained, for example, the high positive supercoiling observed of plasmids isolated from *E. coli* cells when gyrase is inactivated (Lockshon and Morris, 1983), and the isolation of hyper-negative supercoiled plasmid DNA from *top*A mutants which is dependent on the transcription of the *tet*A gene (Pruss and Drlica, 1986). These observations have been incorporated into another model of supercoiling in cells which is dependent upon the tracking of the transcription complex along the DNA molecule. This model has been termed the twin-supercoiled-domain model (Liu and Wang, 1987).

1.5.2 Twin-supercoiled-domain model

The essence of the twin-supercoiled-domain model for transcription is illustrated in figure 1.5.1 for the case of plasmid DNA.

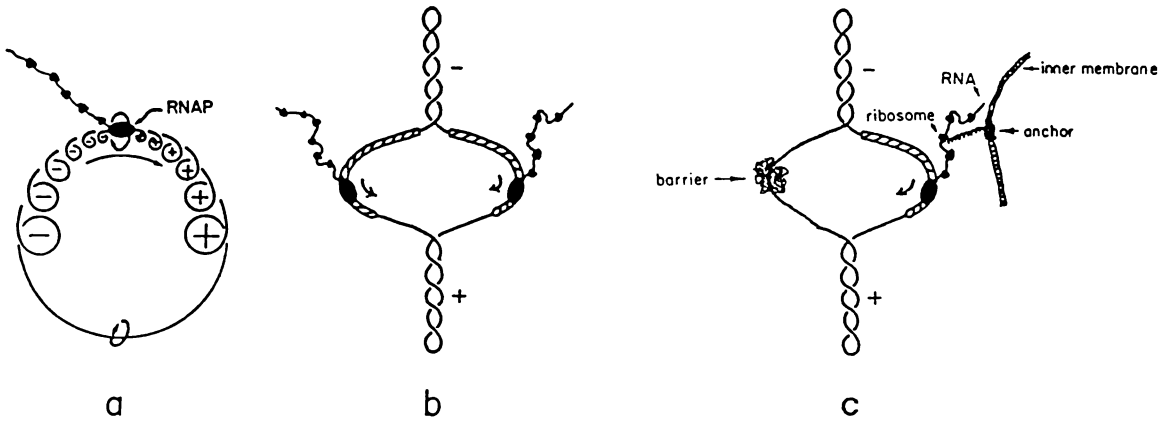


Figure 1.5.1 Models for the formation of twin supercoiling domains during transcription. (a) Shows a single RNA polymerase transcribing along a plasmid (Wu *et al.*, 1988). (b) Shows the transcription of two opposing genes (Wu *et al.*, 1988). (c) Shows the transcription of a single gene with the translated protein anchored in the cell membrane and with a further barrier to diffusion of supercoils being depicted on the left of the plasmid (Lodge *et al.*, 1989).

Figure 1.5.1a depicts a single RNA polymerase molecule transcribing along a circular plasmid DNA. Transcription leads to the rotation of DNA around its helical axis; this rotation is favoured over the rotation of the RNA polymerase elongation complex around the DNA. DNA rotation leads to the positive supercoiling of the DNA ahead of the complex and the negative supercoiling of the DNA behind the complex. For a single transcription complex on a circular template, the opposite supercoils can merge and cancel each other out by rotational diffusion of the DNA segment in between. In such a case, the moving RNA polymerase transcription complex generates two oppositely supercoiled gradients. The degree of supercoiling is highest on regions of DNA nearest to the moving transcription complex.

Figure 1.5.1b depicts a plasmid in which two oppositely orientated genes are being simultaneously transcribed. Merging of the positively and negatively supercoiled regions requires, in addition to the rotation of the connecting DNA, the rotation of at least one of the transcription complexes around the DNA. Therefore transcription would generate two oppositely supercoiled domains if the rotation of the complexes is prevented or subject to a large viscous drag (Wu *et al.*, 1988).

Figure 1.5.1c depicts a plasmid in which one gene is being transcribed. The transcription complex is attached to the bacterial membrane via the translated polypeptide chain and a second barrier to diffusion of supercoils is also depicted. This barrier may be the binding of the plasmid origin to the membrane (Rashtchian *et al.*, 1986). This model was postulated by Lodge *et al.*, 1989 in response to evidence that translation as well as transcription of the *tet*A gene is required to produce highly supercoiled domains and that transcription of the divergent *amp* gene is not required to produce these domains. As the N terminus of the *tet*A protein is thought to be used for membrane insertion (Griffith *et al.*, 1988) the results of Lodge *et al.*, 1989 favour a model in which membrane insertion of the nascent *tet*A protein, still linked to the plasmid DNA by coupled transcription and translation, blocks the transcription complex from turning about the DNA, thereby generating twin domains of supercoiling. When the *tet*A gene was replaced by genes whose protein products do not insert into the membrane, relaxation of supercoiling occurred. Lodge *et al.*, 1989 concluded that divergent transcription alone is not sufficient to cause high negative supercoiling of pBR322 in cells which lack topoisomerase I.

Figures 1.5.1b and 1.5.1c may both occur in the cell. If so, Lodge *et al.*, 1989 suggest that model figure 1.5.1b is more likely to occur in plasmids which contain divergent genes and which are highly actively transcribed (such as *gal*K a non-membrane protein transcribed by the strong *tac* promoter (Wu *et al.*, 1988)) whereas genes which are moderately transcribed (e.g. *tet*A gene) require an effective anchor to maintain twin superhelical domains and this can be provided by anchoring to the inner membrane.

Because eubacterial DNA topoisomerase I and DNA gyrase act differentially on negatively and positively supercoiled domains, the relative rates of at least five processes would influence the degree of supercoiling inside a bacterium:

(1) Transcription which generates positive and negative supercoils at equal rates, and the size, activity, orientation and distribution of transcription units;

(2) Diffusional pathways, which allow the cancellation of positive and negative supercoils and which are dependent on the orientation of transcription units and the attachment of the transcription complex or the DNA to other macromolecules or macromolecular structures;

(3) Binding of RNA polymerase and other DNA binding proteins which relax negative supercoils;

(4) The quantity and distribution of DNA gyrase which catalyses the removal of positive supercoils and introduces negative supercoils. An example of the effect due to lack of DNA gyrase in the cell is shown in figure 1.5.2;

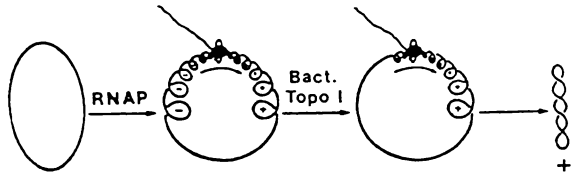


Figure 1.5.2 The effect of gyrase mutants on the supercoiling of transcribing plasmid DNA. Transcription on a relaxed circular DNA template generates positive supercoiling waves ahead of and negative supercoiling waves behind the RNA polymerase elongation complex. In gyrase mutants the positive supercoils are not relaxed whereas DNA topoisomerase I selectively removes the negative supercoils, resulting in a positively supercoiled plasmid (reproduced from Tsao *et al.*, 1989).

(5) The quantity and distribution of DNA topoisomerase I which catalyses the relaxation of negative supercoils.

In normal cells transcription from a strong promoter leads to greater negative supercoiling than transcription from a weak one (Figueroa and Bossi, 1988). This may be due to one of two factors or a mixture of both. First, negative supercoiling may be due to an imbalance in the action of gyrase and topoisomerase I so that the gyrase catalysed relaxation of positive supercoils is faster than topoisomerase I catalysis of negative supercoils (Tsao *et al.*, 1989). This differential rate of relaxation of the twin-supercoiled domains during transcription may explain why intracellular DNA is negatively supercoiled. Second, the magnitude of the supercoiling change appears to correlate with the number of elongating polymerases (Figueroa and Bossi, 1988) therefore Figueroa and Bossi, 1988 have proposed that the binding and transcription of multiple polymerases on a single transcription unit will unbalance topoisomerase activities in favour of relaxing positive supercoils. The outcome is a negative linking difference.

The prevailing view on negative supercoiling in eubacteria has been that DNA gyrase catalyses this reaction. It is plausible however that negative supercoils are generated by the gyrase catalysed relaxation of positive supercoils rather than the active formation of negative supercoils, furthermore this is in agreement with recent studies which show that under physiological conditions, gyrase catalyses the efficient relaxation of positively supercoiled DNA but negative supercoiling activity cannot be detected (Tsao *et al.*, 1989).

Many recent papers suggest that transcription is one of the principal factors affecting intracellular DNA supercoiling (Giaever and Wang, 1988; Tsao *et al.*, 1989; Wu *et al.*, 1988) and in cells with normal levels of topoisomerases supercoiling is likely to be a dynamic process with the formation and decay of localized superhelical domains depending on the relative rates of the various processes. However this localized supercoiling is expected to be highest near the promoters although the complexing of intracellular DNA with macromolecular complexes may retard the dissipation of oppositely supercoiled domains so that supercoiling of the DNA may be affected over a large distance (Tsao *et al.*, 1989). In the case of the negatively supercoiled domain, supercoiling may be sufficiently high to drive structural transitions of certain DNA sequences so that the formation of cruciforms or Z-DNA is favoured. These transitions may in turn affect DNA functions and among other things explain the transcriptional activation of replication and recombination (Frank-Kemenetskii, 1989).

The twin-supercoiled-domain model is applicable not only to eubacteria but has also been shown to occur in eukaryotes, where coupled transcription and translation does not occur, (Giaever and Wang, 1988) and possibly in the halophilic archaebacteria (Giaever and Wang, 1988).

1.6 Temperature effects on DNA and DNA supercoiling

DNA can be denatured by heating and other factors that bring about the dissolution of hydrogen bonding and concomitant unpairing of bases in the double helix leading to the formation of single-stranded DNA. The Tm (midpoint at which DNA melts) is determined by a number of factors including: the guanine plus cytosine content of the DNA; the ionic strength of the buffer in which the DNA is melted; the pH of the buffer; the presence of DNA binding proteins; and for cccDNA the extent of supercoiling. SSC buffer (0.15M NaCl, 15mM trisodium citrate, pH7.0) is usually used when determining the Tm for purified DNA and in this case the thermal denaturation of DNA is directly related to its guanine plus cytosine content by the equation

$$\%G+C = 2.44\ (Tm - 69.4) \qquad \text{(DeLey, 1970)}$$

The following table shows the melting temperature of purified DNA for some thermophilic archaebacteria with *E. coli* as a comparison.

Table 1.6.1 The melting temperarure of purified DNA from *E. coli* and some thermophilic archaebacteria.

Organism	%G+C	Tm	Optimal growth temperature
Escherichia coli	51	90.3°C	37°C
Thermoplasma acidophilum	46.8	88.6°C	55-60°C
Sulfolobus solfataricus	36	84.2°C	70-85°C
Methanobacterium thermoautotrophicum	48.5	89.3°C	65-70°C
Methanothermus fervidus	33	82.8°C	83°C
Pyrococcus woesei	37.5	84.8°C	97-100°C

(%G+C and growth temperatures obtained from Bergeys Manual of Systematic Bacteriology or DSM German Culture Collection of Microorganisms).

It is interesting to note that thermophilic archaebacteria do not always have a particularly high %G+C content and must obviously rely on alternative means to protect their DNA from thermal denaturation.

An increase in ionic strength acts to stabilize DNA against thermal denaturation due to the neutralization of the negatively charged phosphate backbone and the stabilization of hydrogen bonds (Freifelder, 1987) thus a tenfold increase in monovalent cation concentration increases the melting point of DNA by 16.6°C (Lewin, 1987). Polycations would be expected to exert an even greater effect on DNA stability and *Sulfolobus* is known to contain significant amounts of polyamines such as spermidine (Friedman and Oshima, 1989; Kneifel *et al.*, 1986).

Thermoplasma acidophilum lacks a cell wall and therefore cannot stabilize its DNA by maintaining a high internal salt concentration. *Thermoplasma acidophilum* has an intracellular K^+ concentration of 20-50mM (Searcy, 1975). This archaebacterium therefore contains a DNA binding protein, HTa, which acts to protect and stabilize its DNA. Other thermophilic microorganisms have rigid cell walls which can maintain a high intracellular salt concentration and which by itself may be sufficient to protect DNA against thermal denaturation (Searcy, 1986); examples are *Methanobacterium thermoautotrophicum* which contains 1.1M K^+ (Jarrell *et al.*, 1984) and *Methanothermus fervidus* which contains 0.8M K^+ and 0.3M 2'3' cyclic-diphosphoglycerate in addition to eukaryotic histone-like proteins which increase the DNA's thermostability (Sandman *et al.*, 1990).

The binding of proteins to DNA can act to protect against thermal denaturation and

several proteins have been found in thermophilic archaebacteria which appear to have a role in this regard and can increase the point at which melting occurs by 25-40°C (Reddy and Suryanarayana, 1988; Searcy, 1986). Some proteins, notably helix-destabilizing proteins, act to reduce the stability of the DNA helix (Lohman *et al.*, 1988). To my knowledge the role of these proteins in the thermostability of DNA from archaebacteria has not been investigated.

Covalently closed circular plasmid DNA is much more resistant to thermal denaturation than its linear counterpart or genomic DNA and this fact has formed the basis of a plasmid isolation method which uses boiling of a solution to denature and so precipitate all DNA except plasmid cccDNA which does not denature under these conditions (Lev, 1987; Sparks and Elder, 1983). The sign (positive or negative) and extent of supercoiling also appears to be important in the thermostability of plasmid and genomic DNA with the more positively supercoiled DNA being more thermostable presumably due to the tightened helical pitch of the DNA molecule (Kikuchi and Asai, 1984). In this regard it is interesting that the highly thermophilic sulphur metabolizing archaebacteria are the only organisms known which contain a positively supercoiling enzyme (Collin *et al.*, 1988).

Temperature itself has an effect on the supercoiling of DNA due to an increase in the thermal fluctuations of the DNA helix which increases the positive superhelicity of DNA (or relaxes negative supercoils) as the temperature increases (Depew and Wang, 1975). The temperature dependence of the helix rotation angle (twist) was found to be $-0.014° \pm 0.001°/C°$ per base pair (Pullybank *et al.*, 1975). This would mean that plasmid pBR322 (4.36 kb) would contain an extra 10 positive supercoils if the temperature was increased from 20 to 80°C.

1.7 Phylogenetic relationships between archaebacteria and other organisms

All known organisms have been found to fall into three phylogenetically coherent groups, the eukaryotes, the eubacteria, and the archaebacteria (Pace *et al.*, 1986). The division of all life into these three primary kingdoms was first proposed by Woese and Fox, 1977a who used a phylogenetic analysis based upon ribosomal RNA sequence characterization. This phylogenetic analysis further revealed that archaebacteria were closest to the common ancestor of all the kingdoms and this suggests that the archaebacterial phenotype is more ancient and primitive

than the phenotype of other organisms and indeed the environments in which they are found are thought to be the same as that of the early earth (Hahn and Haug, 1986).

Figure 1.7.1 shows an unrooted phylogenetic tree for the three kingdoms based on ribosomal RNA sequences.

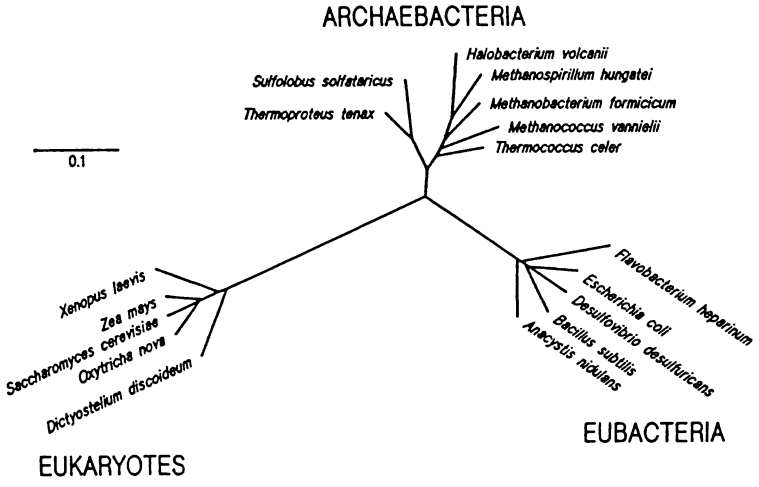


Figure 1.7.1 Unrooted phylogenetic tree for the three urkingdoms constructed from complete sequences of 16S rRNA's. The distance measure (bar) corresponds to 0.1 mutational events per sequence position (reproduced from Woese and Olsen, 1986).

The root of the tree has been left undefined and it appears, under the scheme of figure 1.7.1, that all three kingdoms arose from a single ancestor; the progenote postulated by Woese and Fox, 1977b. The other, and less likely, alternative is that the root lies within the archaebacteria in which case there would have been an early stage in evolution at which all living systems had an archaebacterial character, the eukaryotes and eubacteria then subsequently arising from them (Woese and Olsen, 1986).

There are profound physiological and structural differences between eukaryotes, eubacteria, and archaebacteria which confirms the phylogenetic relationships determined by rRNA sequence studies. These differences are summarised in table 1.7.1. Some of the more important points by which archaebacteria differ or are similar to the other kingdoms, are listed below:

(1) Cell wall structure. Archaebacteria lack muramic acid which is a universal component of the cell wall of eubacteria (Kandler and König, 1985).

(2) Membrane lipid structure. The lipids of archaebacteria are unique from all other organisms in that ester links are replaced by ether links and straight carbon chains are replaced by branched carbon chains (Langworthy, 1985).

(3) Ribosomal RNA. Eukaryotic and sulphur metabolizing archaebacterial rRNA's are highly modified (although the sites of modification differ), whilst the rRNA's of eubacteria and other archaebacteria have much lower levels of modification (Fewson, 1986). Archaebacterial 5S rRNA appears to have both eubacterial and eukaryotic features. The sulphur metabolizing archaebacteria, like eukaryotes, have triphosphorylated 5'-termini whereas the methanogens and halophiles, like the eubacteria, have a 5'-terminal monophosphate (Fox *et al.*, 1982).

(4) DNA dependent RNA polymerases. Archaebacterial RNA polymerases are distinct from those of eubacteria both in structure and function (Gropp *et al.*, 1986). Furthermore the sequence homology between archaebacterial and eukaryotic RNA polymerases is about twice that between archaebacteria and eubacteria (Zillig *et al.*, 1988).

(5) Promoter sequences and RNA translation signals. Translation signals including ribosome-binding sites are comparable in both archaebacteria and eubacteria whereas the consensus sequence of archaebacterial promoters closely resembles that of the eukaryotic polymerase II promoters (Zillig *et al.*, 1988).

In the literature there is a state of considerable flux concerning the exact phylogenetic relationships between the three kingdoms and their positions in relation to the putative progenote and amongst the archaebacteria themselves. The most widely published controversy has been the claim by Lake that the sulphur metabolizing archaebacteria (renamed the eocytes) and the eukaryotes form one group while the halobacteria and eubacteria form a second group (retermed the photocytes) which leaves the methanogens as a third group (this group is left with the name archaebacteria) (Lake *et al.*, 1985; Lake, 1986; Lake, 1988). Lake bases his claim on two main lines of evidence. Firstly, on analysis of three-dimensional ribosome structure which was used to probe evolutionary divergences (Lake *et al.*, 1985) and second, on an analysis of rRNA sequences using the evolutionary parsimony method which is relatively unaffected by changes in mutation rates (Lake, 1988). Further evidence put forward by Lake *et al.*, is the occurrence of related biochemical processes in halobacteria and eubacteria including essential components of

Table 1.7.1 Distribution of shared (s) and unique (u) feature designs between archaebacteria (AB), eubacteria (EB) and eukaryotes (EK) (reproduced from Zillig *et al.*, 1988).

Presence of feature in: +, all or most members of kingdom; (+), certain phyla of kingdom; ± intermediate or divergent character of feature; (?) interpretation questionable. The archaebacteria show 3 or 4, the eubacteria 5 and the eukaryotes 8 – 10 of the unique features contained in this list; the archaebacteria 12 or 13, the eubacteria 8 and the eukaryotes 6 or 7 of the shared features contained in this list

Feature	AB	EB	EK
Murein	–	(+)u	–
Outer membrane	–	(+)u	–
7S RNA	+?	–	(+)u
Isopranyl ether lipids	+u	–	–
Ester lipids	–	+s	+s
S^0 · reduction	(+)s	(+)s	–
SO_4^{2-} reduction	(+)s	(+)s	–
S^0 oxidation	(+)s	(+)s	–
Methanogenesis	(+)u	–	–
Nitrogen fixation	(+)s	(+)s	–
Photosynthesis (chlorophyll)	–	(+)u	–[a]
Operons	+s	+u	–
mRNA splicing	–	–	+u
tRNA splicing	(+)s	–	(+)s
CCA end of tRNA encoded	–	(+)u	–
fMet-tRNA	–	+	–
First base pair in initiator tRNA A · U	+s	–	+s
Aphidicolin-sensitive DNA polymerase	+s	–	+s
Reverse gyrase	(+)u	–	–
Promoter of pol II type	+s	–	+s
Promoter of eubacterial type	–	+u	–
80S ribosomes	±	–	+(s)
70S ribosomes	(+)s	+s	–
5.8S rRNA	–	–	+u
EFTu ADP-ribosylation possible	+s	–	+s
Ribosome binding sites	+s	+s	–
RNA polymerase of archaebacterial type	+s	–	+s
RNA polymerase of eubacterial type	–	+u	–
Specialized RNA polymerases	–	–	+u
Capping of mRNA			+u
Poly(A) tailing of mRNA			+u
Nucleus	–	–	+u
True chromatin (nucleosomes)	–	–	+u
Chromosomes			+u
Multi-cellular organisation	–	±	(+)u
Somatic differentiation	–	–(?)	(+)u
Cell division by septum formation	±s	+s	–
Cell division by other mechanisms e.g. constriction	±s	–	+s

[a] The chlorophyll found in eukaryotic cells is in chloroplasts of eubacterial origin.

the photosynthetic apparatus such as carotenoids. This was taken to mean that photosynthesis could be a primitive property of both groups hence the term 'photocytes'.

Lake's phylogenetic scheme has been strongly criticized in many papers. The essence of these criticisms can be found in Woese and Olsen, 1986 regarding phylogenetic determinations using ribosome morphology, and in Gouy and Li, 1989 regarding phylogenetic analysis using the evolutionary parsimony method on small rRNA sequences. According to Woese and Olsen, 1986, Lake's analysis, using ribosome morphology to determine phylogenetic relationships, rests on five assumptions:

(1) That morphological features of ribosomes are reliable and primary phylogenetic determinants;

(2) That certain ribosome morphological characteristics have a particular distribution among the archaebacteria, eukaryotes, and eubacteria;

(3) That changes in the individual features are sufficiently rare that a parsimony analysis is appropriate;

(4) That quantitative variations in size of these features represent an ordered transition and;

(5) That analysis of other data yields the same conclusions as that obtained from ribosome morphology.

Woese and Olsen state that "most or all of these assumptions appear to be incorrect" and "that ribosome morphology is a phylogenetic determinant of questionable significance". Gouy and Li also criticise Lake's scheme and show that phylogenetic analysis based on rRNA sequences supports the traditional archaebacterial tree (Gouy and Li, 1989).

Another deviation from the accepted three kingdom division of all organisms has been recently proposed. In this scheme another kingdom has been added, called the archezoa, which are eukaryotic-like organisms totally lacking in mitochondria and peroxisomes (Cavalier-Smith, 1989). Another recent hypothesis, which is gaining support from several lines of evidence, is the phylogenetic closeness of the archaebacterial and eukaryotic kingdoms. The phylogenetic distance between these two groups appears to be shorter than that found for the archaebacterial-eubacterial or the eukaryotic-eubacterial relationships (Iwabe *et al.*, 1989; Pühler *et al.*, 1989).

1.7.1 Phylogenetic relationships amongst archaebacteria

The archaebacteria are a diverse group of organisms and display a wide variety of phylogenetic characteristics. Different organisms within the archaebacteria may be: autotrophic (*Pyrodictium*) or heterotrophic (*Thermoplasma*); obligate aerobes (*Sulfolobus*) or anaerobes (*Methanobacterium*) or facultative (*Acidianus*); acidophilic (*Sulfolobus*) or alkalophilic (*Natronococcus*); halophilic (*Halobacterium*) or able to grow under very low ionic strengths (*Thermoplasma*); and extremely thermophilic (*Pyrococcus*) or mesophilic (*Methanogenium*) (König and Stetter, 1989). Archaebacteria show only three basic phenotypes; methanogenic, halophilic, and thermophilic sulphur-metabolizing, however these phenotypes do not necessarily reflect phylogenetic relationships. On the basis of 16S ribosomal RNA sequences (Woese and Olsen, 1986) and hybridization of ribosomal RNA's (Klenk *et al.*, 1986), the archaebacteria have been divided into three phylogenetically coherent groups which are:

(1) The methanogenic bacteria and their relatives including the extreme halophiles and the genus *Thermoplasma*;

(2) The *Thermococcales* and;

(3) All other thermophilic sulphur-metabolizing archaebacteria.

A phylogenetic tree which shows the relationships between these three groups is shown in figure 1.7.2.

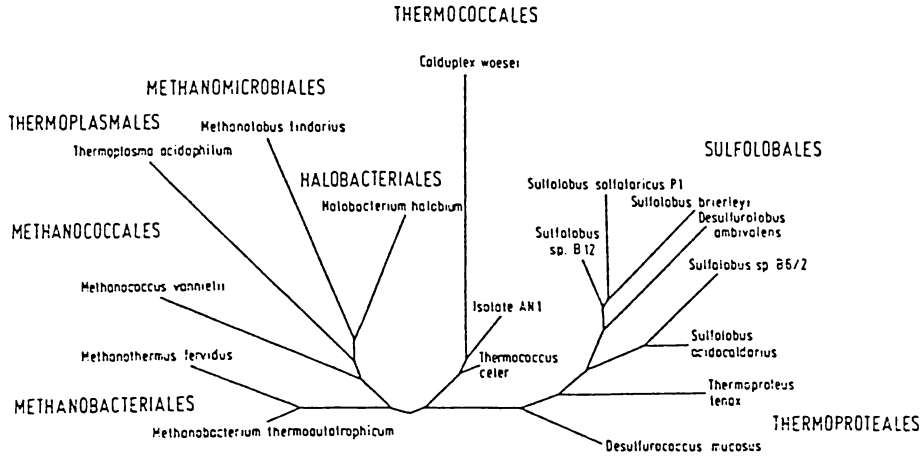


Figure 1.7.2 Phylogenetic tree of the archaebacteria obtained by analysis of hybridization homology of ribosomal RNA's of 17 species of archaebacteria (from Klenk *et al.*, 1986). Note that *Caldoduplex woesei* has been renamed *Pyrococcus woesei*.

In this scheme the extreme halophiles arise out of the *Methanomicrobiales*, one of the three major methanogen orders. *Thermoplasma* likewise groups with the methanogens, and within this group, on the line leading to the halophile-methanomicrobiales cluster. The group containing the aerobic *Sulfolobales* and the anaerobic *Thermoproteales* is relatively closely related and phenotypically homologous when compared to the phenotypically diverse and phylogenetically deep groupings found in the methanogenic branch. Within the sulphur-metabolizing branch of the archaebacteria the aerobic *Sulfolobales* appear to be more rapidly evolving than the anaerobic *Thermoproteales* from which they are thought to have arisen (Woese and Olsen, 1986).

The group comprising of the *Thermococcales* appears to be phylogenetically intermediate between the other two groups (Zillig *et al.*, 1987). It would seem to represent the most slowly evolving of the archaebacterial lines (Woese and Olsen, 1986) and it branches from the other archaebacteria near the root of the archaebacterial tree. However, on the basis of the 16S ribosomal RNA sequence from *Thermococcus celer* it appears possible that this group is more closely aligned to the methanogenic branch of the archaebacteria than to the alternate sulphur-metabolizing branch (Achenbach-Richter *et al.*, 1988). Furthermore the root of the archaebacterial tree at which the eubacterial and eukaryotic lineages intersect is also found between the *Thermococcales* and the other sulphur-metabolizing archaebacteria (Achenbach-Richter *et al.*, 1988). In relation to this thesis this is an interesting proposal as the distribution of reverse gyrase is found in all sulphur-metabolizing archaebacteria including the *Thermococcales* (Collin *et al.*, 1988). This would presuppose that reverse gyrase was the ancestral type I topoisomerase and that other type I topoisomerases found in the methanogen branch (for example topoisomerase I from *Thermoplasma* Forterre *et al.*, 1989) may be subsequent derivatives of this enzyme.

The picture of archaebacterial evolution that emerges is that the ancestral archaebacterium was an extreme thermophile and that for one of the archaebacterial groups the ancestral phenotype has remained fundamentally unchanged whereas on the other major group a variety of phenotypes has arisen; organisms that can live at lower temperatures and generate energy by producing methane, and (from these) organisms that live in highly saline environments

(Achenbach-Richter *et al.*, 1988).

The methanogenic group of organisms comprises five orders:

(1) *Methanobacteriales*, (2) *Methanococcales* and (3) *Methanomicrobiales*. These three orders are obligate anaerobes and are distinguished from all other organisms in that they alone produce methane as the major catabolic product. They contain unique coenzymes such as coenzyme M, F_{420}, F_{430}, methanopterin, and methanofuran (Boone and Mah, 1989). They are found in a wide variety of habitats including marshes, anaerobic sediments, insect guts, and the rumen of cattle and sheep (Fewson, 1986).

(4) *Halobacteriales*. This order comprises the extremely halophilic archaebacteria. They are aerobic or facultatively anaerobic and require at least 1.5M NaCl for growth although most strains grow best at 3.5 - 4.5M NaCl. Colonies are various shades of red due to the presence of C50 carotenoids. This group occurs ubiquitously in nature where the salt concentration is high i.e. in salt lakes and seas, soda lakes, and salterns (Grant and Larsen, 1989).

(5) *Thermoplasmales*. Members of this order lack a cell wall, they are obligatory thermoacidophilic growing at a temperature of 55-59°C and a pH of 1-2. These organisms have been isolated from self-heating coal refuse piles (Langworthy and Smith, 1989) and a Japanese hot spring (Stetter and Zillig, 1985).

The second group of archaebacteria comprises only one order the *Thermococcales*.

(1) *Thermococcales*. Members of this order are spherical and often occur as diplococci. They are motile with a polar tuft of flagella. They utilize different carbon sources by sulphur respiration to form H_2S from S° (Zillig *et al.*, 1983). These organisms are found in marine solfataras, or in hot springs containing NaCl in environments of neutral pH and 80-103°C (Zillig, 1989a). This order comprises the genera; *Thermococcus*, *Pyrococcus*, and isolate AN1. Isolate AN1 was isolated from an 80°C, pH 6-7.5 hot pool in Rotorua New Zealand. This organism, which is closely related to *Thermococcus celer*, is dependent on sulphur for growth and has a sodium requirement of 10-200mM NaCl. Isolate AN1 is an irregular coccoid of 0.5-2.0um with a polar tuft of flagella and displays optimum growth at a temperature of 75-80°C and a pH of 7.3.

The third group of archaebacteria comprises two orders, the *Thermoproteales* and the *Sulfolobales*.

(1) *Thermoproteales*. Members of this order may be rod, disc or spherical cells. They are anaerobic and may grow either chemolithotrophically using CO_2 as a sole carbon source or by the sulphur respiration of various organic substrates as is the case for the *Thermococcales*. The *Thermoproteales* occur world-wide in solfataric hot waters and mudholes at temperatures of up to 100°C or in superheated submarine solfataric environments of 103°C or higher (Zillig, 1989b). This order comprises the genera: *Thermoproteus, Thermofilum, Desulfurococcus, Staphylothermus, Pyrodictium*, and *Thermodiscus*.

(2) *Sulfolobales*. Members of this order are coccoid and irregularly lobed. They are aerobic or facultatively anaerobic. They may grow aerobically by the oxidation of sulphur or sulphur containing compounds or alternatively (in the case of *Acidianus*) by the anaerobic reduction of sulphur with H_2. These organisms are thermoacidophilic growing above 45°C (Normally 70-75°C) with an optimum pH for growth of about 2. Members of the *Sulfolobales* can be isolated world-wide from acidic solfataric hot waters or boiling mudholes (Stetter, 1989). This order comprises two genera, *Sulfolobus* and *Acidianus*. The genus *Sulfolobus* comprises two species *S. acidocaldarius* and *S. solfataricus*. *S. solfataricus* (the reverse gyrase enzyme of which is studied in this thesis) are non-motile lobed spherical cells of 0.8-2um in diameter. They differ from *S. acidocaldarius* by an RNA polymerase whose components exhibit different molecular weights and by a difference in optimum growth conditions where *S. solfataricus* grows at 80-85°C and pH 3.5-5.0 while *S. acidocaldarius* grows at 70°C and pH 2-2.5 (Zillig *et al.*, 1980).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Plasmid isolation and purification

2.1.1 pBR322 cleared lysate isolation

DAY 1

10ml of LB medium (see appendix I) containing 0.5% glucose and 15ug/ml tetracycline was inoculated with a loopful of *E. coli* C600 containing plasmid pBR322. This was grown overnight (O/N) at 37°C.

DAY 2

75ml of LB medium containing 0.5% glucose and 50ug/ml ampicillin was inoculated with an O/N culture and grown at 37°C in a shaking incubator (200rpm) until an OD_{600} of 0.6 was reached (~4hr). This growing culture was then added to 1.5l of LB medium containing 0.5% glucose (3x500ml in 2l fluted flasks). When an OD_{600} of 0.6 was reached, chloramphenicol was added to a final concentration of 20ug/ml. The culture was incubated for 16hr at 37°C in a shaking incubator (200rpm).

DAY 3

The following steps were carried out at 0°C. The culture was centrifuged for 20min at 10,000g and the pellet resuspended in 15ml of ST solution (10% sucrose, 50mM Tris/HCl pH8.0) and left on ice for 30min. 500ul of lysozyme solution (10mg/ml in H_20) was added and the tube rotated and left for 5min. 2.6ml of 0.5M EDTA (pH7.8) was added, the tube rotated and left for 5min. 7.5ml of Triton solution (2% Triton X-100, 50mM Tris/HCL pH8.0, and 62.5mM EDTA pH8.0) was added and the tube rotated and left for 30min. The lysate solution was centrifuged at 48,000g for 20min at 0°C.

The supernatant was carefully tipped into a sterile weighed beaker and 9.77g of CsCl and

0.8ml of ethidium bromide (10mg/ml) added to each 10g of the supernatant to give a final density for the solution of 1.55g/ml.

This was centrifuged at 250,000g for 20hr at 15°C in a Beckman TL100 centrifuge with a TLA.100 fixed angle rotor. Tubes were illuminated using ultra-violet light at 366nm (Comag Universal UV lamp) and the plasmid band removed. Plasmid bands were pooled and rerun using the aforesaid conditions.

After the second CsCl centrifugation the plasmid band was removed and ethidium bromide extracted by adding an equal volume of 90% isopropanol saturated with CsCl. After mixing, the two phases were allowed to separate and the isopropanol (upper phase) removed with a Pasteur pipette. This step was repeated until the isopropanol remained colourless.

The plasmid preparation was then either:

(a) dialysed against 2x1l of TE buffer (10mM Tris/HCl pH8.0, 1mM EDTA pH8.0), or

(b) diluted 4x with MilliQ water, 7.5M ammonium acetate added to half the original volume then 2.5 volumes of ethanol added. This was left for 10min at room temperature, and centrifuged at 16,000g for 30min. The DNA precipitate was resuspended in 2.5ml of TE buffer.

The plasmid solution was mixed with an equal volume of phenol (prepared by the method of Maniatis *et al.*, 1982). The phases were separated by low speed centrifugation and the aqueous (upper phase) removed. Residual phenol was removed from the aqueous phase containing the plasmid by extraction with ether. Ether and the plasmid preparation were mixed, the phases allowed to separate, and the ether (top phase) removed. This extraction was repeated 3x or until the plasmid solution remained clear.

The plasmid preparation was blown with compressed air until ether could not be detected by smell.

Finally the plasmid solution was precipitated using the method described by Crouse and Amorese (BRL, Focus 9.2, 1987). 0.5 volumes of 7.5M ammonium acetate was added to the solution followed by 2.5 volumes of 100% ethanol. The solution was mixed by inversion and left at room temperature for 10min followed by centrifugation at 16,000g for 30min. The supernatant was decanted and the pellet dried under vacuum for 30min. The plasmid pellet was resuspended in an appropriate volume of TE buffer and stored at -20°C.

2.1.2 pBR322 Birnboim isolation (alkaline lysis)

This procedure is a modified version of the alkaline lysis protocol used by Birnboim and Doly, 1979 to isolate plasmid DNA.

DAY 1

2.5ml of *E. coli* C600 containing plasmid pBR322 was inoculated into 500ml of TB medium (see appendix I) containing ampicillin (25ug/ml) in a 2l flask and incubated O/N at 37°C with vigorous shaking (200rpm).

DAY 2

Cells were pelleted at 10,000g for 20min (4°C) and resuspended in 12ml of 25mM Tris/HCl pH8.0, 10mM EDTA, and 50mM glucose. After incubation on ice for 20min the resuspended cells were slowly added to 24ml of 0.2M NaOH and 1% SDS and gently but thoroughly mixed (adding the cell suspension to the alkali seemed to improve lysing efficiency). After incubation on ice for 10min the cell mix was added to 15ml of 3M NaAcetate pH4.6, mixed gently and left to incubate on ice for 20min. The solution was centrifuged at 12,000g for 30min (4°C) and the supernatant decanted into a sterile centrifuge tube leaving the white precipitate behind. To the solution was added 7.5M ammonium acetate to a final concentration of 2.5M and after incubation on ice for 10min the solution was centrifuged at 12,000g for 10min (4°C). The supernatant was decanted into a sterile centrifuge tube, 2.5 volumes of 100% ethanol added, and the solution incubated on ice for 10min followed by centrifugation at 12,000g for 30min (4°C). The precipitate was resuspended in 5ml of TE buffer (10mM Tris/HCl pH8.0, 1mM EDTA pH8.0) and a 10mg/ml RNase solution (pancreatic RNase A, Sigma, dissolved in 10mM Tris/HCl pH7.5, 15mM NaCl, boiled for 15min and allowed to cool slowly) free of DNase added to a final concentration of 10ug/ml. The RNase containing solution was incubated at 37°C for 20min and prepared for CsCl centrifugation by adding 9.77gm CsCl and 0.8ml of ethidium bromide (10mg/ml) for each 10g of solution to give a final density of 1.55g/ml. The rest of the procedure followed that used for the cleared lysate method.

2.2 Viral DNA isolation and purification

2.2.1 Growth and isolation of knotted P4 phage DNA

DAY 1

5ml of *E. coli* C117 was grown O/N at 37°C on modified Luria broth. *E. coli* C117 is a P2 lysogen and was obtained from L.F. Liu, Johns Hopkins School of Medicine, Baltimore.

DAY 2

An equal volume of modified Luria broth was added to the O/N culture and incubated for 1-1.5hr. After incubation 4ul of $2.5x10^8$ pfu/ml P4 phage was added to the culture and the phage allowed to absorb by sitting at room temperature for 10min. 4l of modified Luria broth was divided into 8x500ml batches and 1ml of the phage/bacteria solution added to each batch. 2l flasks containing 500ml of phage/bacteria solution were incubated at 37°C with vigorous aeration (200rpm) and without plugs. The absorbance at 600nm was monitored every 30min from the time of inoculation. After 2.5hr, readings were taken more frequently until the readings started to drop (4.5hr), this indicated lysis of the cells. When lysis began 5ml of 0.5M EGTA pH8.0 was added to each flask to prevent phage reabsorption. Shaking was continued for 1hr and 1ml of chloroform then added to each flask to complete lysis.

The lysed bacteria and phage P4 solution was pelleted at 12,000g for 20min (4°C) to remove cell debris and to the supernatant was added NaCl to 0.5M, polyethylene glycol 8000 to 10%, and 10g of $MgCl_2$ per four litres. This solution was incubated O/N at 4°C.

DAY 3

The solution was centrifuged at 16,300g for 30min (4°C) and the pooled pellets extracted three times with P4 diluent (1% ammonium acetate, 80mM $MgCl_2$, and 10mM Tris/HCl pH7.2) to a final volume of 80ml. The supernatants from each extraction were pooled and centrifuged at 50,000g for 4hr (4°C). The pellet was resuspended in 6ml of P4 diluent. This resuspension was difficult and was left O/N at 4°C.

DAY 4

To each gram of phage solution was added 0.632g of CsCl and this was ultracentrifuged

in polycarbonate tubes at 245,000g for 18hr (8°C) using a Beckman TLA.100 rotor in a Beckman TL100 ultracentrifuge. After centrifugation, two main milky-coloured bands were apparent. The top band was complete phage particles while the bottom band contained tailless P4 heads with knotted DNA.

These bands were removed using a 20 gauge needle and each put into a separate ultracentrifuge tube. These tubes were topped off with P4 diluent to give a dilution factor of 5 or greater and mixed well. The tubes were ultracentrifuged at 270,000g for 1.5hr (4°C) in a Beckman TL100 ultracentrifuge. After pelleting the complete phage particles were resuspended in P4 diluent and stored at 4°C for use in further preparations. The tailless P4 heads containing knotted P4 DNA were resuspended in 3ml of TN buffer (10mM Tris/HCl pH8.0, 100mM NaCl) and extracted twice with phenol followed by extraction four times with ether. Knotted P4 DNA was ethanol precipitated and stored in TEN buffer (10mM Tris/HCl pH8.0, 1mM EDTA, and 100mM NaCl).

2.2.2 Growth and isolation of ssM13 phage DNA

DAY 1

10ml of *E. coli* JM101 was grown O/N at 37°C on 2xYT medium (see appendix I).

DAY 2

The O/N culture was added to 150ml of prewarmed 2xYT and grown at 37°C with no shaking until an absorbance at 600nm of 0.3 was reached. At this stage M13 phage was added (4.5ml at $1x10^{11}$ pfu/ml) to give a multiplicity of infection of 10 viral particles to 1 cell. Incubation was continued for 5hr at which stage the titre reached $1x10^{11}$ to $5x10^{12}$ pfu/ml. The culture was centrifuged at 8000g for 20min (4°C) to pellet the cells and the supernatant decanted into a sterile centrifuge tube. To the supernatant was added NaCl to 0.5M, polyethylene glycol 8000 to 10%, and $MgCl_2$ to 0.25%, and left O/N at 4°C.

DAY 3

The solution was centrifuged at 16,300g for 30min (4°C) and the pellet resuspended in 2ml of TEM buffer (10mM Tris/HCl pH8.0, 1mM EDTA, and 50mM $MgCl_2$). 0.632g of CsCl was added per g of phage solution and centrifuged at 245,000g for 18hr (8°C) in a Beckman TL100

ultracentrifuge. The single milky coloured band was removed with a large gauge needle, diluted 5x in TE buffer (10mM Tris/HCl pH8.0, 1mM EDTA pH8.0), and recentrifuged at 270,000g for 2hr (4°C) to pellet the virus. The pellet was resuspended in 500ul of TE buffer and extracted twice with phenol followed by extraction four times with ether. M13 DNA was ethanol precipitated and stored in TE buffer at -20°C. This DNA was tested to ensure its single-stranded nature by incubation with S1 nuclease. This treatment, at 1 unit S1 nuclease per ug M13 DNA, totally degraded the single DNA band normally observable on an agarose gel whereas it had little effect on double-stranded pBR322.

2.2.3 Growth and isolation of T4 phage DNA

The plaque forming units per ml (pfu/ml) of a cell lysate containing T4 was determined by serially diluting the lysate 10 fold from 10^{-4} to 10^{-14}. 100ul of the diluted lysate was added to 100ul of *E. coli* B grown into the logarithmic phase (4 x 10^8 cells/ml) on phage broth pH7.2 (see appendix I). The T4, *E. coli* mix was incubated for 10min at 37°C, 3ml of soft agar (see appendix I) added and mixed and then quickly spread out onto T phage nutrient agar plates (see appendix I). These plates were incubated at 37°C until plaques became visible and the number of plaques per plate counted.

The growth and isolation of T4 phage was carried out as described in Clowes and Hayes, 1968.

A 0.2ml O/N culture of *E. coli* B was added to 20ml of phage broth and incubated at 37°C in a shaking incubator until an OD_{600} of 0.125 (1x10^8 cells/ml) was reached.

T4 was added to a final concentration of 10^9 pfu/ml.

Incubation at 37°C was continued until lysis by clearing was observed, this took 2-3hr.

2 drops of chloroform was added and shaking continued for 1min.

The culture was left to stand 10min and then centrifuged at 6000g for 20min to remove bacterial debris.

T4 phage was sedimented from the supernatant by centrifuging at 23,000g for 1hr.

The precipitated T4 phage were resuspended in 1ml of T4 phage buffer (Na_2HPO_4 7g/l, KH_2PO_4 3g/l, NaCl 5g/l, 1M $MgSO_4$ 1 ml/l, 1M $CaCl_2$ 100ul/l) and left to stand O/N.

100ul of virus preparation was further purified by running on a 10-30% (w/v) sucrose gradient at 17,000g for 20min. Fractions were collected, peak fractions retained and dialysed against T4 phage buffer.

2.3 DNA modifying enzymes and restriction enzymes

2.3.1 The reverse gyrase assay

The standard reverse gyrase assay in most experiments followed that used by Nakasu and Kikuchi, 1985 where 1ul of reverse gyrase solution was added to 9ul of assay mix containing; 50mM Tris/HCl pH7.5 (20°C), 0.2M NaCl, 10mM $MgCl_2$, 1mM ATP, 1mM spermidine, 1mM DTT, and 0.4ug negatively supercoiled pBR322. After centrifugation at 6,000g for 1min to spin the contents to the bottom of the eppendorf tube (1.5ml capacity), incubation was carried out in a 75°C waterbath for 10min. The reaction was stopped by cooling to room temperature followed by the addition of 1/5 volume (2ul) of 50% glycerol, 5% SDS, 50mM EDTA, and 0.05% bromophenol blue. If the enzyme solution was too concentrated it was diluted with 50mM Tris/HCl pH7.5, 50ug/ml bovine serum albumin, and 1mM spermidine.

In later experiments the standard assay was slightly changed by the replacement of 50mM Tris/HCl pH7.5 (20°C) with 50mM MOPS/NaOH pH6.6 (20°C) and the use of a 80°C incubation temperature rather than 75°C.

2.3.2 Eco R1 endonuclease

DNA (pBR322 or P4 DNA) was cut on several occasions with Eco R1 using the following protocol. A stock solution of Eco R1 (Bethesda Research Laboratories) at 10 units/ul was diluted 10 fold in storage buffer (50mM Tris/HCL pH7.2, 0.3M NaCl, 0.5mM EDTA, 5mM mercaptoethanol, 5mM EGTA, 0.2% (v/v) Triton X-100, 500ug/ml BSA, and 50% glycerol) to give 1 unit/ul. 2 units of Eco R1 was added to 1ug of DNA and the volume made to 9ul with milliQ H_2O. 1ul of REact 3 (Bethesda Research Laboratories) was added to bring the volume to 10ul and to give a final reaction mix containing 50mM Tris/HCl pH8.0, 10mM $MgCl_2$, and 100mM NaCl. This was incubated for 1hr at 37°C and gave total cutting of both pBR322 and P4 DNA.

2.3.3 S1 nuclease

DNA (pBR322 and M13) was cut with S1 nuclease on several occasions using the following protocol. A stock solution of S1 nuclease (Bethesda Research Laboratories) at 1000 units/ul was diluted 500 fold in S1 nuclease storage buffer (20mM Tris/HCl pH7.5, 50mM NaCl, 0.1mM zinc acetate and 50% glycerol) to give 2 units/ul. 1 to 10 units of S1 nuclease was added to 1ug of DNA and a 2x concentration of S1 nuclease assay mix added to give a final reaction mix containing 30mM sodium acetate pH4.6, 50mM NaCl, 1mM zinc acetate, and 5% glycerol. This was incubated for 30min at 37°C and the reaction stopped by heating at 70°C for 5min or by adding 1/5 volume of a loading solution comprised of 50% glycerol, 5% SDS, 50mM EDTA, and 0.05% bromophenol blue.

Under these conditions 0.5ug ssM13 DNA was completely degraded by 1 unit of S1 nuclease and 0.5ug of negatively supercoiled pBR322 was 90% digested to linear and open circular forms by 10 units of S1 nuclease.

2.3.4 T4 DNA ligase

T4 DNA ligase was used to ligate the cohesive ends of knotted P4 DNA by the following protocol; 1ul of a stock solution of T4 DNA ligase (Bethesda Research Laboratories) at 1 unit/ul was used to ligate 8ug P4 DNA in DNA ligase reaction buffer (5x) to give a final reaction mix containing 50mM Tris/HCl pH7.6, 10mM $MgCl_2$, 1mM ATP, 1mM DTT, and 50ug/ml bovine serum albumin. This was incubated at 16°C for 2hr to give total ligation of P4 DNA as evidenced by a lack of degradation of the knotted substrate at high temperatures (80°C, 10min).

2.4 Agarose gel electrophoresis

Agarose gel electrophoresis of DNA was normally performed on a mini horizontal gel electrophoresis unit which was a modification of that described in Maniatis *et al.*, 1982. The gel platform size was 10x10cm with a gel volume of 50ml and a buffer volume of 450ml. If a greater degree of separation was required a BioRad DNA subcell was used with a gel platform size of 15x15cm, a gel volume of 120ml, and a buffer volume of 1500ml. Electrophoresis power packs to

run the units were from Bethesda Research Laboratories (Model 250) and ATTO Corporation (Model AE-8400).

1% agarose gels were prepared by melting the agarose (Ultra Pure Agarose, electrophoresis grade, Bethesda Research Laboratories) in 1xTBE (89mM Tris base, 89mM boric acid, and 3mM EDTA pH8.3) using a heating mantle. The agarose solution was cooled to 50°C in a waterbath and poured onto the gel platform which had plastic dams sealed by 1% agarose at both ends to restrict the agarose solution to the gel platform. A teflon comb was inserted at one end and the gel allowed to set. Electrophoresis buffer was poured over the gel and the comb and dams removed.

The electrophoresis unit with gel was filled with 1xTBE to 0.5cm over the level of the gel. Samples were loaded into the wells and electrophoresis carried out at constant voltage for 400V.hr at a maximum of 100V. On most occasions electrophoresis was performed O/N at 25V.

For two-dimensional gels the first dimension was run as described above and the gel and buffer removed to a container where 3.5ul of 1mg/ml ethidium bromide (Sigma) was added to a total of 500ml of buffer and gel (final concentration of 0.007ug/ml ethidium bromide). This was left for 1hr at room temperature, placed on the gel platform at a 90° orientation to the original position, sealed with 1% agarose and electrophoresed for 200V.hr at 50 volts.

One and two dimensional gels were stained for 20min in 0.2ug/ml final concentration of ethidium bromide with gentle agitation. Destaining of the gel was accomplished by soaking the gel for 20min in 500ml of 1mM $MgSO_4$ with gentle agitation. This was repeated with fresh 1mM $MgSO_4$ for a further 20min and the gel visualized by ultra-violet light at 366nm with a Fotodyne U.V. Transilluminator. Gels were photographed with a Polaroid CU-5 Land camera with a yellow filter and mounted on an industrial accessory mount. Polaroid 665 film was used with an exposure of F4.7 and a time of 15sec. Development of the film was for 35sec and negatives were fixed in 18% $NaSO_3$ for at least 10min and rinsed in water for 10min.

2.4.1 Laser scanning densitometry of agarose gels

Densitometry of agarose gels was achieved with a photographic negative of the gel and using an LKB 2202 Ultroscan Laser Densitometer connected to a Commodore PC20-II computer with GS-365 Data System software (Hoefer Scientific Instruments).

For the determination of relative topoisomerase activity the conversion of form I DNA to other topoisomers was measured by determining the quantity of form I DNA and comparing it with the quantity of form I DNA in a control where no topological conversion had occurred. A reduction in 80% of form I DNA compared with a control was taken to equal 1 unit of activity under standard conditions. The eleven tracks in a mini gel could be examined in one scan using the auto scan function and the relative densities of form I DNA from the tracks directly compared using the GS-365 Data system software.

2.5 Growth and preparation of bacterial cells to screen for reverse gyrase activity

Many of the bacteria used in this project are currently held in the Thermophile Research Unit Culture Collection (TRUCC). Other bacteria were obtained from the German Collection of Microorganisms (DSM) and from the American Type Culture Collection (ATCC).

Where possible, bacteria were grown in the recommended media which can be found in the German Collection of Microorganisms, Catalogue of Strains, 3rd ed, 1983 and the American Type Culture Collection, Catalogue of Strains I, 15th ed, 1982.

Strains of *Thermoproteus*, *Desulfurococcus*, and AN1 were grown on media described in appendix I and prepared anaerobically. Anaerobic bacteria were maintained in a viable condition by serial transfer in Hungate tubes. These were incubated for 2 days and then stored for up to 3 weeks at RT.

Aerobic bacteria were normally stored at -20°C in their growth media with the addition of 15% glycerol. *Sulfolobus* strains were maintained by adjusting the pH to 6.5 and storing in the dark at room temperature.

2.5.1 The preparation of bacteria for determining reverse gyrase activity

Cells were grown to stationary phase at their optimum temperature and harvested by centrifugation at 13,000g for 20min. Pellets were resuspended in 10x w/v of lysis buffer (50mM Tris/HCl pH7.5, 0.6M NaCl, 1mM spermidine, 1mM phenylmethyl-sulfonyl fluoride (PMSF), and 5mM mercaptoethanol).

0.5-1ml of the suspended cells were lysed by sonication (Kontes, micro-ultrasonic cell

disrupter set at 10.5 watts). 10min of sonication was usually sufficient to disrupt over 90% of the cells. The lysed cells were centrifuged at 13,000g for 15min and the supernatant stored in 20% glycerol at -20°C until required for assay.

1ul of the supernatant was used for determining the presence of reverse gyrase activity in the assay procedure (see 2.3.1).

Assay conditions were in some cases varied where the intracellular conditions of bacterial species were at variance with the standard assay conditions. All mesophilic bacteria were assayed for reverse gyrase at their optimum growth temperature and at 75°C. The assay mix was modified for the *Halobacterium* sp to include mixes containing 1M NaCl, 3M NaCl, 0.5M KCl, 1.5M KCl, and 3M KCl. The assay mix was modified for *Thermoplasma* to include pH's of 5.6 (60°C) and 5.5 (60°C).

2.6 Purification and characterization of reverse gyrase from the *Thermococcus* like isolate AN1

AN1 cells were obtained from a large scale culture of this organism in a 600l stainless steel fermenter vessel (Bragger, 1990). 6 litres of a well grown culture were inoculated into the vessel and the cells incubated for 38hr at 80°C and at a pH of 7.2. Cells were harvested using a continuous flow Sharples AS16 centrifuge and stored at -70°C. This procedure also centrifuged out sulphur in the medium so that the final pellet contained both sulphur particles and cells.

Unless otherwise stated, all procedures for AN1 reverse gyrase purification were carried out at room temperature (16-24°C). Column fractions were stored at 0-5°C between purification steps. All buffer solutions used during purification were made up with milliQ water, an ultra pure water with a resistivity of 16-18 megaohms, produced by the milliQ Water Purification System (Millipore).

All buffers used for column chromatography were degassed before use and, in the case of buffers used with FPLC columns, filtered through a 0.45uM filter (Millipore).

2.6.1 Preparation of a cell-free extract and $(NH_4)_2SO_4$ precipitation

15gm of AN1 cell pellet was thawed from -70°C and resuspended in 60ml of lysis buffer

containing 50mM Tris/HCl pH7.5, 1mM spermidine, 1mM PMSF, and 10mM mercaptoethanol. Sulphur was removed by filtration through Whatman No 1 filter paper and cells lysed by sonication on ice for approximately 20min (in 5min bursts) using a Dynatech Sonic Dismembrator (series 300, Artek) with a power setting of 60% and using a titanium tip with a diameter of 19mm. Cells were at least 90% lysed as determined by observation with phase contrast microscopy.

The solution was centrifuged at 195,400g for 2hr at 4°C in a Prepspin 50 (MSE) centrifuge with a 8x50ml rotor (43114-130 rotor). The clarified supernatant was decanted into a clean centrifuge tube and 25.4g $(NH_4)_2SO_4$ added to 60% saturation. The solution was stirred at 4°C for 30min and then centrifuged in a Sorvall centrifuge (RC-5B) with a Sorvall GSA rotor at 16,270g for 30min at 4°C. The pellet was drained and redissolved in 20ml of lysis buffer containing 0.5M NaCl. This solution was dialysed in 3,500mw cutoff dialysis tubing (Spectrapor). Dialysis was carried out overnight at 4°C and against 2x2l volumes of resuspension buffer. The solution was ultrafiltered to 17ml (26mg/ml) by using an Amicon ultrafiltration apparatus with a YM10 membrane.

2.6.2 Fractogel TSK gel permeation chromatography

A Fractogel TSK HW-55(F) column (Merck) (39.5 x 5cm, bed volume 775ml, void volume 150ml) was equilibrated with a buffer solution containing 0.5M ammonium acetate, 0.5M NaCl, 1mM spermidine, 5mM mercaptoethanol, and 1mM PMSF adjusted to a pH of 6.5 with acetic acid. This column was run at a flow rate of 200ml/hr using a peristaltic pump (Pharmacia P-3). The 17ml sample was loaded onto the column and 10ml fractions collected using a LKB 2111 multirac fraction collector. The eluent absorbance was monitored at 280nm, by a Cecil CE272 linear readout ultraviolet spectrophotometer (Cecil instruments). Active fractions from the run were combined and the 200ml solution (20 fractions) concentrated by ultrafiltration (YM10 membrane, Amicon) to 50ml.

2.6.3 Blue Dextran Agarose affinity chromatography

The AN1 solution (50ml) was dialysed with stirring at 4°C against two 2l volumes of 20mM Tris/HCl pH7.0, 0.1M NaCl, 5mM mercaptoethanol, and 1mM spermidine (Blue Dextran Agarose

starting buffer) over 14hr. The AN1 solution (51ml, 2.5mg/ml) was loaded onto a Blue Dextran Agarose (Sigma) column (25ml) at a flow rate of 45ml/hr and washed with 50ml of Blue Dextran Agarose starting buffer. Reverse gyrase was eluted at 50ml/hr with a ten column volume (250ml) salt gradient to 1.0M NaCl. The eluent was monitored at 280nm with a Cecil CE272 linear readout ultraviolet spectrophotometer and 6.3ml fractions collected using a LKB 2111 multirac fraction collector. In order to elute remaining activity the column was further washed with 50ml of starting buffer at pH7.5 and 1.5M NaCl. Active fractions from the salt gradient (63ml) and material from the final wash (50ml) which contained activity, were pooled and concentrated by ultrafiltration (YM10 membrane, Amicon) to 38.5ml (0.24mg/ml).

2.6.4 Anion exchange FPLC

The 38.5ml AN1 solution was dialysed against anion exchange starting buffer (20mM Tris/HCl pH7.5, 1mM spermidine, and 5mM mercaptoethanol) as described in the previous section. A MonoQ HR5/5 anion exchange FPLC column (Pharmacia) was equilibrated in starting buffer at 1ml/min (HPLC pump, Waters Assoc) and the sample loaded onto the column using a 50ml superloop (Pharmacia). After a 5ml wash with starting buffer, reverse gyrase was eluted with a 30min linear salt gradient to 0.5M NaCl in the same buffer using a model 660 solvent programmer (Waters Assoc) to develop the gradient. 1ml fractions were collected using a Frac-100 fraction collector (Pharmacia) and the eluent absorbance monitored at 280nm by a Lambda-Max UV detector (1cm path length, model 481, Waters Assoc). Active fractions were pooled to give a 5ml (1.1mg/ml) reverse gyrase solution.

2.6.5 Cation exchange FPLC

The 5ml of pooled reverse gyrase activity from anion exchange FPLC was dialysed overnight against 2l of MonoS starting buffer (50mM MES/NaOH pH6.7, 0.1M NaCl, 1mM spermidine, and 5mM mercaptoethanol). A MonoS HR5/5 cation exchange FPLC column was equilibrated at 1ml/min in the above buffer and the sample loaded onto the column using a 50ml superloop (Pharmacia). After a 5ml wash the bound protein was eluted with a 30min linear salt gradient to 0.6M NaCl in the same buffer. The eluent was monitored at 280nm and 1ml fractions

were collected as described in section 2.6.4. Active fractions were pooled and solid $(NH_4)_2SO_4$ added to give a final concentration of 0.9M in 7ml of solution (total 0.28mg protein).

2.6.6 Hydrophobic interaction FPLC

A Phenyl-Suparose HR 5/5 hydrophobic interaction FPLC column (Pharmacia) was equilibrated with 50mM Tris/HCl pH6.8, 0.85M $(NH_4)_2SO_4$, and 1mM spermidine at 0.5ml/min (HPLC pump, Waters Assoc). The 7ml sample was loaded onto the column using a 50ml superloop (Pharmacia) and after washing with 5ml of equilibration buffer, reverse gyrase was eluted with a 40min linear gradient to 50mM Tris/HCl pH6.8 and 1mM spermidine. The eluent was monitored at 280nm and 1 ml fractions collected as described in section 2.6.4. Active fractions (3ml) were pooled and dialysed in 3500MW cutoff dialysis tubing (Spectrapor) against 10mM MOPS/NaOH pH7.2, 1mM spermidine, 1mM DTT, and 2% mannitol. The active material was freeze dried and stored at 4°C. The freeze dried powder, which retained full activity, was later reconstituted and used in characterization of AN1 reverse gyrase.

2.7 Growth and Harvesting of *Sulfolobus solfataricus*

S. solfataricus was grown either in batch culture in 2l flasks (500ml per flask) incubated in a Gallenkamp orbital incubator (Gallenkamp, England) at 75°C and 120rpm, or in a New Brunswick 10l glass fermenter, model Cf-14 (New Brunswick Scientific Co, N.J.) immersed in a 75°C waterbath with a layer of polystyrene beads floating on the water to prevent evaporation. Aeration of the fermenter was accomplished by an air pump feed to the fermenter through a 0.2um filter. In all cases cells were grown in the medium recommended for *S. solfataricus* by the DSM. Due to the low yield of *Sulfolobus* cells in the fermenter (possibly due to the inclusion of stainless steel components in the system) this procedure was not used after two runs and the majority of cells were produced in 2l flasks using a 5% inoculum of cells (late log phase).

Cells were harvested at late log phase (OD_{600} ~0.5). After cooling the culture to room temperature and adjusting the pH to 6.2-6.8 with 2M Tris, the cells were centrifuged at 10,410g for 20min (GSA rotor) in a Sorvall RC-5B refrigerated superspeed centrifuge. Cells harvested from the fermenter were concentrated prior to centrifugation by using an Amicon DC10LA ultrafiltration

system with an Amicon Diaflo hollow fibre cartridge (H5 MP 01-43) with a molecular weight cutoff of 0.1uM.

Cells were frozen in liquid nitrogen and stored at -20°C until required. Cell growth was monitored at 600nm and, in some cases, were counted using a Thoma-Hawksley counting chamber (0.02mmx1/400mm^2/square) with a volume of 5x10^{-8}ml per square. The cell doubling time during log phase growth was determined by the equation:

$$MGT = \frac{0.301t}{\log_{10}X_t - \log_{10}X_0} \qquad \text{(Brock, 1974)}$$

MGT = mean generation time
t = length of time from X_0 to X_t
X_0 = number of cells at time 0
X_t = number of cells at time t

2.8 Purification of *S. solfataricus* reverse gyrase - Scheme I

The comments made in the introduction of section 2.6 in purification of the AN1 reverse gyrase enzyme also apply to this section.

2.8.1 Preparation of the cell free extract, Polymin P and $(NH_4)_2SO_4$ precipitations

38g of cell pellet was thawed and suspended in 300ml of lysis buffer containing 50mM Tris/HCl pH7.5 (20°C), 0.6M NaCl, 1mM spermidine, 1mM PMSF, and 5mM mercaptoethanol. The cells were lysed by sonication on ice with a Dynatech Sonic Dismembrator (series 300, Artek) set at a power setting of 60% and using a titanium tip with a diameter of 19mm. After sonication for 15min in 5min bursts the lysate was slightly acidic. This was adjusted to a pH of 7.5 using 2M Tris. The lysate was centrifuged at 195,400g for 2hr at 4°C in a Prepspin 50(MSE) centrifuge with a 8x50ml rotor (43114-130 rotor). The clarified supernatant was stored at 4°C while the pellet was resuspended in lysis buffer and sonicated on ice for a further 15min (5min bursts). This material was centrifuged as before and the supernatant pooled with the initial supernatant to give a total volume of 336ml.

Polymin P (Sigma) to a final concentration of 0.3% was added to the supernatant by dropwise addition of 20% Polymin P solution (pH7.5) and gently mixed for 15min on ice. The

precipitate was removed by centrifugation in a Sorvall RC-5B centrifuge with a GSA rotor at 10,410g for 30min at 4°C. Solid $(NH_4)_2SO_4$ was added to the supernatant to 60% saturation with constant stirring and stirring continued for 30min at 4°C before centrifugation for 30min as above. The pellet was redissolved in 100ml of CM Sepharose starting buffer comprising 20mM MES/NaOH pH6.1, 1mM spermidine, 50mM NaCl, and 5mM mercaptoethanol. The solution was dialysed (3,500mw cutoff membrane, Spectrapor) with stirring at 4°C against 2x2l volumes of CM Sepharose starting buffer.

2.8.2 CM Sepharose CL-6B Chromatography

The dialysed *S. solfataricus* sample was diluted with 400ml of CM Sepharose starting buffer to give a protein concentration of 3.5mg/ml. The sample was loaded onto a CM Sepharose CL-6B (Sigma) cation exchange column (900ml, 15x90cm) preequilibrated in starting buffer at a flow rate of 3.3ml/min using a peristaltic pump (Pharmacia P-3). The column was washed with 2l of starting buffer and developed at 7ml/min with a 5 column volume salt gradient to 1M NaCl, the eluent being monitored at 280nm with a Cecil CE272 linear readout ultraviolet spectrophotometer (Cecil instruments) and 40ml fractions collected using a LKB2111 multirac fraction collector. Active fractions were combined and concentrated by ultrafiltration (YM10 membrane, Amicon) to 60ml. This solution was dialysed with stirring at 4°C against 2x2l of Heparin Agarose starting buffer comprising 50mM Tris/HCl pH7.5, 50mM NaCl, 1mM spermidine, and 1mM DTT.

2.8.3 Heparin Agarose chromatography

The *S. solfataricus* solution was loaded onto a 50ml (2.6x9.4cm) Heparin Agarose (Sigma) column, equilibrated with Heparin Agarose starting buffer, at a rate of 1.8 ml/min (Pharmacia pump P-3). The column was washed with 200ml of starting buffer and reverse gyrase eluted with a 5 column volume linear salt gradient to 1M NaCl. Eluent monitoring and fraction collecting were as described in the previous section but with 10ml fractions being collected. The most active fractions were combined and concentrated by ultrafiltration with a YM10 membrane (Amicon). The concentrated solution was loaded onto a 40ml desalting column containing Bio-

Gel P-6DG desalting gel (BioRad) and the buffer exchanged for the anion exchange starting buffer (50mM Tris/HCl pH7.5, 1mM spermidine, and 0.5mM DTT).

2.8.4 Anion exchange FPLC

A MonoQ HR 10/10 anion exchange FPLC column (Pharmacia) was equilibrated in starting buffer at 3ml/min (HPLC pump, Waters Assoc.). One half of the reverse gyrase sample (7ml) obtained after Heparin Agarose chromatography, was loaded onto the column using a 50ml Pharmacia superloop. After a 40ml wash with equilibration buffer, reverse gyrase was eluted with a 10 column volume (80ml) linear salt gradient to 0.5M NaCl in the same buffer. 2.5ml fractions were collected using a FRAC-100 fraction collector (Pharmacia) and the eluent absorbance monitored at 280nm with a Lambda-Max UV detector (1cm path length, model 481, Waters Assoc.).

Active fractions were combined and stored at 4°C while another anion exchange FPLC run was undertaken on the second half of the sample obtained from Heparin Agarose chromatography. For both runs the elution profiles appeared identical and reverse gyrase eluted under the same salt conditions. Active fractions from both runs were combined and the 82ml solution concentrated by ultrafiltration followed by buffer exchange into MonoS starting buffer (20mM MOPS/NaOH pH6.5, 1mM spermidine, 200mM NaCl, and 0.5mM DTT).

2.8.5 Cation exchange FPLC

The sample from anion exchange FPLC was applied to a MonoS HR 5/5 cation exchange FPLC column (Pharmacia). After column washing, the bound protein was eluted with a 30ml linear salt gradient to 0.5M NaCl at 1ml/min. The eluent was monitored at 280nm and 1ml fractions were collected as described in the previous section. Active fractions were combined and solid $(NH_4)_2SO_4$ added to give a concentration of 1M.

2.8.6 Hydrophobic interaction FPLC

The above sample was loaded onto a Phenyl-Superose HR 5/5 hydrophobic interaction FPLC column (Pharmacia) equilibrated in 50mM Tris/HCl pH6.8, 1M $(NH_4)_2SO_4$, 1mM spermidine

and 0.5mM DTT, at 0.5ml/min. The sample was loaded using a 50ml Pharmacia superloop and after a 5ml wash a linear gradient (20ml) of decreasing $(NH_4)_2SO_4$ concentration to 0M $(NH_4)_2SO_4$ was applied to the column.

The eluent was monitored at 280nm and 1ml fractions were collected as described in section 2.8.4. At this stage 3 fractions contained the peak of activity and these were pooled. To one half of this material was added an equal volume of sterile AnalR glycerol and the solution stored at -20°C. The second half of this material was desalted on a 10ml Bio-Gel P-6DG desalting column (BioRad) against 10mM MOPS/NaOH pH7.5 and 5% mannitol, freeze dried and stored at 4°C. These two fractions were subsequently tested for purity by SDS-PAGE.

2.9 Purification of *S. solfataricus* reverse gyrase - Scheme II

The comments made in the introduction of section 2.6 in purification of the AN1 reverse gyrase enzyme also apply to this section. However the initial steps of this scheme (up to dye-binding chromatography) were carried out at 4°C rather than room temperature.

2.9.1 Preparation of the cell free extract, Polymin P and $(NH_4)_2SO_4$ precipitations

S. solfataricus cells (44gm wet weight) were thawed from -20°C to room temperature and 260ml of lysis buffer added (lysis buffer consisted of 50mM MOPS/NaOH pH7.0, 1.2M NH_4Cl, 1mM EDTA, and 1mM PMSF). After resuspension of the cells in this buffer the solution was sonicated for 40min in 10min bursts (5min between bursts) using a Sonicator Ultrasonic Processor (Model W-380, Heat Systems Ultrasonic Inc.) with a 13mm titanium tip. The sonicator was set at 80% output with a 50% duty cycle and 5sec pulses. It was estimated, using phase contrast microscopy, that over 80% of the cells were lysed by this process. The lysate was centrifuged at 16,270g for 10min at 4°C in an RC-5B Sorvall centrifuge with a GSA rotor. The supernatant was stored at 4°C while the pellet consisting of cell debris and whole cells was resuspended in 50ml of lysis buffer and resonicated for 15min under the aforesaid conditions. After centrifugation as before, the two supernatants were pooled to give a volume of 340ml and a concentration of 15.5mg/ml protein. A 16% Polymin P solution was added gradually and with constant stirring to bring the supernatant to 0.36% Polymin P. After further gentle stirring for

15min on ice the solution was centrifuged at 10,410g for 30min at 4°C using a RC-5B Sorvall centrifuge with a GSA rotor. The supernatant was centrifuged using a Prepspin 50(MSE) ultracentrifuge with a 8x50ml rotor (43114-130 rotor) at 195,400g for 2hr at 4°C. To the clarified supernatant was added $(NH_4)_2SO_4$ to a final concentration of 70%. This was stirred on ice for 30min and centrifuged at 16,270g for 30min at 4°C (Sorvall RC-5B centrifuge with a GSA rotor). The pellet was resuspended in 100ml of 50mM MOPS/NaOH pH7.0, 1mM DTT, 1mM EDTA, and 35% $(NH_4)_2SO_4$. After stirring for 30min on ice the suspension was centrifuged as before and the 130ml of supernatant diluted with 100ml of 50mM MOPS/NaOH pH7.0, 1mM DTT, 1mM EDTA, and 3M NaCl to give final concentrations of approximately 1.2M NaCl and 0.8M $(NH_4)_2SO_4$. The solution was clarified by further centrifugation at 16,270g for 20min at 4°C and the supernatant stored at 4°C.

2.9.2 Hydrophobic interaction chromatography

A Phenyl Sepharose CL-4B (Sigma no P-7892) hydrophobic interaction column (50ml, 2.6x9.4cm) was equilibrated with 50mM MOPS/NaOH pH7.0, 1mM DTT, 1.2M NaCl, and 0.8M $(NH_4)_2SO_4$. One half of the above sample was loaded onto the column at a rate of 0.83ml/min (Pharmacia peristaltic pump P-3) and washed with 250ml of equilibration buffer. Another wash with 250ml of 50mM MOPS/NaOH pH7.0, 1mM DTT, 1mM EDTA, and 0.25M NaCl was carried out followed by a further wash in 200ml of the same buffer with the addition of 30% ethylene glycol. The column was developed with a 500ml linear gradient of 30-60% ethylene glycol in the aforesaid buffer. Eluent was monitored at 280nm with a 1cm pathlength flow cell (model UA-5 absorbance/fluorescence monitor with a type 6 optical unit, ISCO) and 10ml fractions collected using a 2112 Redirac fraction collector (LKB Bromma). Some protein remained bound to the column at this stage and was eluted with a final wash in 50mM MOPS/NaOH pH7.0, 1mM DTT, 1mM EDTA, and 1% Triton X-100.

Active fractions from the gradient were pooled and stored at 4°C while a second run was initiated. This run followed the methodology of the first and produced essentially the same elution profile. Active fractions from the second run were pooled and combined with that of the first run. This solution was ultrafiltered using a YM10 Amicon membrane and dialysed against

2x2l of dye-binding starting buffer consisting of 50mM MES/NaOH pH6.0, 100mM NaCl, 1mM DTT, 10mM $MgCl_2$, and 1mM spermidine, using 3,500mw cutoff dialysis tubing (Spectrapor).

2.9.3 Dye-binding chromatography

The sample, equilibrated with the appropriate buffer, was loaded onto a Cibacron Navy F2R (Centre for Protein and Enzyme Technology, Australia) dye-binding column (50ml, 2.6x9.4cm) at 0.83ml/min and washed in 150ml of starting buffer. Reverse gyrase was eluted with a 500ml linear salt gradient to 1M NaCl in the same buffer, the eluent being monitored at 280nm and 10ml fractions collected using the equipment described in the previous section. Active fractions were combined and concentrated by ultrafiltration with a YM10 membrane (Amicon) and solid $(NH_4)_2SO_4$ added to a final concentration of 1M.

2.9.4 Hydrophobic interaction FPLC

A Phenyl-Superose HR 5/5 hydrophobic interaction FPLC column (Pharmacia) was equilibrated with 50mM MOPS/NaOH pH7.0, 50mM NaCl, 1mM DTT, 1mM spermidine, and 1M $(NH_4)_2SO_4$ at 0.5ml/min using a HPLC pump (Waters Assoc.). One third of the above sample was loaded onto the column using a 50ml superloop (Pharmacia) and washed with 5ml of equilibration buffer. The bound protein was then eluted with a 30ml linear $(NH_4)_2SO_4$ gradient to 0M $(NH_4)_2SO_4$ in the same buffer, the eluent being monitored at 280nm and 1ml fractions being collected as described in section 2.8.4. The most active fractions were combined and stored at 4°C while two repeat runs were carried out on the remaining solution from the dye-binding step. The elution profiles of all three runs were identical with reverse gyrase activity eluting from the column at the same position on every run. Active fractions were combined and concentrated by ultrafiltration with a YM10 membrane (Amicon) to a final volume of 2ml (0.83mg/ml).

2.9.5 Gel permeation FPLC

A Superose 12 HR 10/30 analytical gel permeation column (bed volume ~24ml) (Pharmacia) was equilibrated with 50mM MOPS/NaOH pH7.0, 0.5M NaCl, 1mM spermidine, 1mM DTT, and 5% glycerol at 0.4ml/min using an HPLC pump (Waters Assoc.). 200ul of solution

was loaded onto the column and eluted, the eluent being monitored and 0.4ml fractions collected as described in section 2.8.4. Successive chromatographic runs were performed in order to process material from the hydrophobic interaction column. Active fractions were pooled then ultrafiltrated using a Centricon-10 microconcentrator (10,000MW cutoff, Amicon). This material was diluted 50x in 20mM MOPS/NaOH pH7.0, 0.5mM DTT, 1mM spermidine, 20mM NaCl, and 200mM mannitol, reconcentrated and finally freeze dried to yield a powder which contained highly active reverse gyrase activity.

2.10 Partial purification of *Bacillus caldovelox* topoisomerase I

A partially purified topoisomerase activity was obtained from a fraction comprising the supernatant of a 50% $(NH_4)_2SO_4$ cut produced using the following steps (Patchett, 1988). *B. caldovelox* cells (1.5 Kg wet weight) were thawed from -70°C to 20°C over 6hr and transferred to a 10l stainless steel container. Six litres of extraction buffer containing 7.5ml Triton X-100, 1.5g lysozyme.HCl (Sigma), 75mg DNase (Sigma), and 11.2g KCl, in 16.6mM MES/NaOH buffer, pH5.5 (20°C), was added and the slurry stirred while heating to 37°C (15min). Stirring at this temperature was continued for 40min and the lysate cooled to 4°C (15min) and centrifuged at 14,700g for 30min at 4°C. The supernatant pH was adjusted to 8.25 (20°C) with the addition of 300ml of 1.5M EPPS/NaOH pH9.5, bringing the total volume to 6.85l. 1.2Kg of $(NH_4)_2SO_4$ was added gradually with constant stirring over 15min to bring the supernatant to 30% saturation. After further gentle stirring for 35min the solution was centrifuged at 10,200g for 20min at 20°C. 0.807Kg of $(NH_4)_2SO_4$ was added as before to the 6.44 litres of supernatant to bring the solution to 50% saturation. This was centrifuged as above and the pellet separated from the supernatant.

2.10.1 Anion exchange FPLC

A MonoQ HR 10/10 anion exchange FPLC column (Pharmacia) was equilibrated in 20mM bis-Tris/HCL pH6.1, 5% sorbitol, 1mM spermidine, and 5mM mercaptoethanol, at 3ml/min (HPLC pump, Waters Assoc). 10ml of the 50% $(NH_4)_2SO_4$ supernatant was desalted into the starting buffer using a 40ml desalting column containing Bio-Gel P-6DG desalting gel. 8ml of the desalted material, at a concentration of 20mg/ml was injected onto the column. After a two

column volume wash with the starting buffer (16ml) the column was developed with a 10 column volume (80ml) linear salt gradient to 1M NaCl in the same buffer. 3ml fractions were collected using a FRAC-100 fraction collector (Pharmacia) and the eluent absorbance monitored at 280nm by a Lambda-Max UV detector (1cm path length, model 481, Waters Assoc). The five most active fractions were combined and the 15ml solution desalted into starting buffer as previously described. The desalted solution was freeze-dried and the powder, comprising in the main, sorbitol, stored at 4°C.

2.11 Protein electrophoresis

2.11.1 SDS polyacrylamide gel electrophoresis

The discontinuous buffer system of Laemmli (1970) was used with the inclusion of 1% SDS in the gel and buffers.

Samples were prepared for electrophoresis by adding 1/4 volume of 4x Laemmli sample buffer (63mM Tris/HCl pH6.8, 10% glycerol, 2% SDS, 5% mercaptoethanol, and 0.0013% bromophenol blue) and boiling the sample for 3min in a boiling water bath. Samples were finally spun at 12,000g for 5min to eliminate any precipitates. Protein samples in high salt solutions were processed by precipitation of the protein as follows. An ice cold solution of 50% tricholoroacetic acid (TCA) was added to the protein solution to a final concentration of 10%. The solution was left for 30min or O/N at -20°C and centrifuged at 12,000g for 15min. The supernatant was decanted and the precipitate resuspended in 10-50ul of 1x Laemmli sample buffer, the sample buffer turned yellow due to the acidic nature of the precipitate. 1N NaOH was added to the sample in 1ul lots until the solution became neutral (turned blue). Heating and centrifugation of the sample was carried out as previously described. Discontinuous Laemmli gels contained the ingredients shown in table 2.11.1.

All ingredients except ammonium persulphate and TEMED were combined and degassed before adding the two catalysts. Separating gels were poured, overlaid with isobutyl alcohol, and allowed to set for 1hr at room temperature. The top of the separating gel was rinsed thoroughly in distiled water to remove the overlay and unpolymerised acrylamide, and the gel

was drained of excess water. At this stage gels could be stored at room temperature under humid conditions for up to two weeks or used immediately. The stacking gel was made in the same way as the separating gel and poured on top of the separating gel the top of which was first thoroughly dried. A comb was placed in the stacking gel solution and left until polymerization of the stacking gel was complete (~20min). The comb was removed and the wells rinsed with distiled water and drained before mounting the gel on the cooling core and inserting into the electrophoresis buffer tank.

Table 2.11.1 Constituents of discontinuous polyacrylamide gels. Acrylamide and bis-acrylamide were electran grade reagents from BDH (Poole, England). Tris was high purity reagent grade 'Trizma base' from Sigma. SDS (special quality) was obtained from Boehringer Mannheim.

Solution	Stacking gel	Separating gels		
	4%	7.5%	10%	20%
Acrylamide/Bis (30%T, 2.67%C)	1.3ml	11.3ml	15ml	30ml
10%SDS	100ul	450ul	450ul	450ul
1.5M Tris/HCl pH8.8	-	11.3ml	11.3ml	11.3ml
0.5M Tris/HCl pH6.8	2.5ml	-	-	-
MilliQ H_20	6.1ml	21.8ml	18.1ml	3.3ml
10% ammonium persulphate	50ul	225ul	100ul	100ul
TEMED	10ul	22.5ul	10ul	6ul
Total volume	10ml	45ml	45ml	45ml

0.75mm thick gels were normally used and were run on a Protean II Slab Cell system (BioRad) at ~15°C with a current of 13mA during stacking and 18mA during protein separation. Under these conditions homogeneous gels took 4-5hr to run and gradient gels took 6hr to run.

Gradient gels (10-20% polyacrylamide) were made using the same solutions and procedures described above except that a gradient former (model 385, BioRad) was used to prepare a separating gel whose polyacrylamide concentration was linear from 10% at the top of the gel to 20% at the bottom of the gel. The procedure for this is outlined below: Solutions were prepared and catalysts added to one half total volumes of 10% and 20% polyacrylamide solution. The two solutions were quickly poured into the gradient mixer, the 20% polyacrylamide solution into the reservoir chamber and the 10% polyacrylamide solution into the mixing chamber. The

valve between the two chambers was opened and solution from the mixing chamber allowed to slowly flow under gravity between two glass plates with 0.75mm spacers. The solution was loaded from the bottom of the glass plates and took ~10min to load. Polymerization started to occur at ~20min and was essentially complete in 1hr. Before loading the polyacrylamide solution into the glass sandwich a layer of 0.05% SDS was added (1cm) to prevent oxygen inhibition of gel polymerization.

Staining of protein bands on polyacrylamide gels was achieved using either a Coomassie Blue R250 stain or a more sensitive silver stain.

The Coomassie Blue R250 staining mixture contained 1g of the dye (Sigma) dissolved in 500ml methanol to which 100ml glacial acetic acid and 400ml water were added. Gels were stained, with gentle shaking, for 1hr before destaining in 40% methanol and 10% acetic acid (3 washes of 1hr each) and were stored in 5% acetic acid (Andrews, 1986).

The silver staining procedure used was based on that of Merril *et al.*, (1982) and is described below. The times given in this silver staining procedure are suitable for 0.75mm gels.

Step	Reagent	Time
1	Fixative A[a]	1hr or O/N
2	Fixative B[b]	30min
3	Fixative B	30min
4	Oxidizer[c]	8min
5	MilliQ H_20	8min
6	MilliQ H_20	8min
7	Silver Nitrate[d]	30min
8	MilliQ H_20	1min
9	Developer[e]	10sec
10	Developer	~2min
	(removed when solution begins to take on a brown colour)	
11	Developer	5-10min
	(until developed)	
12	5% acetic acid	5min
13	MilliQ H_20	-

[a] 40% methanol, 10% acetic acid (v/v)

[b] 10% ethanol, 5% acetic acid (v/v)

[c] 0.25g $K_2Cr_2O_7$, 35.4ul HNO_3, 250ml MilliQ H_2O (made fresh)

[d] 0.51g $AgNO_3$, 250ml MilliQ H_2O (made fresh)

[e] 59.3g Na_2CO_3, 2.5ml 37% formaldehyde, 2l milliQ H_2O.

For all steps 250ml of solution was used and the gel gently agitated in a glass dish. Polyacrylamide gels were stored in sealed plastic bags with a small amount of water. Gels were mounted on a light box and photographed with Polaroid 665 film using a Polaroid CU-5 Land camera (F5.6, 1/30 second exposure).

SDS-PAGE was also run using the PhastSystem (Pharmacia) which includes the PhastSystem separation and control unit and the PhastSystem development unit.

PhastGel gradients 10-15 (Pharmacia) with PhastGel SDS buffer strips (Pharmacia) were used for all separations, and gels stained with PhastGel Blue R (Pharmacia) or silver stained using the procedures and protocols outlined in the PhastSystem Owners Manual (Pharmacia).

2.11.2 Isoelectric focusing

Agarose isoelectric focusing gels were prepared by adding 1% (0.2g) agarose IEF (Pharmacia) and 12% (2.4g) D-sorbitol (Sigma) into 18.3ml of milliQ H_2O. After mixing, the solution was boiled to dissolve the agarose and the solution cooled to 75°C in a waterbath. 6% (1.2ml) of 40% (w/v) Servalyt ampholytes (AG 3-10, Serva) were slowly added to the solution and then poured into a preheated (65°C) casting frame (10x12x0.15cm) open at the top and consisting of a polyester backing sheet (Gel-Fix for Agarose, 42961, Serva) held to a glass supporting plate by a few drops of water and a second glass plate separated from the supporting plate by two 0.75mm teflon spacers. The casting frame containing molten agarose IEF solution was allowed to cool to room temperature and the gel hardened at 4°C for 1hr or O/N before use.

Isoelectric focusing apparatus was prepared by lowering the agarose gel onto a Pharmacia flatbed (FBE-3000) with n-decane between the gel and the flatbed to serve as a heat exchanger. Wicks (1x10cm) were placed on the gel at either end, the cathode (-) wick containing 1M NaOH solution and the anode (+) wick containing 10mM H_2SO_4 solution. Both wicks were well blotted before use so that excess water which accumulated during the run (especially at the anode end) was absorbed. Samples (5-50ul) were applied to the gel using Serva applicator strips. These were removed half way through the run once the sample had been absorbed by the gel.

Isoelectric focusing of the gel was carried out for 1.25hr at 5 watts with a voltage limit of 1500 volts using an ISCO electrophoresis power supply (model 494). A temperature of 18°C was maintained on the flatbed by using a FH15 Grant heater/circulator, and a FC25 Grant cooler.

Agarose gels were stained for protein by the Coomassie Blue R250 method described in section 2.11.1. After destaining, gels were transferred to water for 15min and then dried at 37°C with the backing sheet still attached.

Isoelectric focusing was also used with the PhastSystem (Pharmacia) which includes the PhastSystem separation and control unit and the PhastSystem development unit. PhastGel IEF 3-9 gels were run and stained with PhastGel Blue R at 50°C as set out in the PhastSystem Owners Manual (Pharmacia).

2.12 Protein determination

For most protein determinations a modified dye binding method of Bradford (1976) was used. A stock solution was prepared by dissolving 100mg of Coomassie Blue G250 (Pierce) in 50ml 100% ethanol. To this was added 100ml 85% (w/v) orthophosphoric acid and the solution stirred for 30min. The Coomassie Blue reagent was made fresh every two weeks and consisted of 30ml of stock solution and 200ml H_2O. The assay procedure involved the mixing of 3ml of dye reagent with 10ul of sample containing 0.5-5mg/ml protein with the absorbance of the solution being read at 595nm up to 1hr after mixing. The protein concentration of unknown solutions was determined from the standard curve for bovine serum albumin which gave a linear relationship within 0.5-5mg/ml with an r value of typically 0.99. Unknown samples with 5-50mg/ml or 50-500ug/ml protein could also be tested by adjusting the volume of unknown mixed into the reagent to 1ul or 100ul respectively. This volume change appeared to have no effect on the sensitivity or accuracy of the assay.

A second method for protein determination was at times used, especially for the more crude protein preparations, and this was protein estimation by extinction at 260 and 280nm (Dawson, 1974). The extinction of an appropriately diluted protein solution was measured at 260 and 280nm and the ratio of E_{280}/E_{260} calculated. Using this ratio the proportion of nucleic acid in the protein solution could be determined to give a factor for the calculation of the absorbance

due to protein so that: Protein concentration (mg/ml) = Extinction at 280nm x Factor. A 1cm light path is assumed and the factor at 0% nucleic acid (E_{280}/E_{260} = 1.75) is 1.12.

2.13 Nucleoid procedures

2.13.1 Production of *E. coli* nucleoids

Isolation of nucleoids from *E. coli* was carried out essentially as described by Stonington and Pettijohn (1971) with changes suggested by Worcel and Burgi (1972). 1ml of an O/N culture of *E. coli* K12 F^- was added to 25ml of M9CA media (see appendix I). This was incubated in a shaking incubator at 180rpm and 37°C until an OD_{600} of 0.25 ($2x10^8$ cells/ml) was reached.

The cells were quickly chilled to 2°C in an ethanol/ice bath and centrifuged at 10,000g for 10min at 2°C.

The pellet was resuspended at 0°C in 200ul of solution A and transferred into an eppendorf tube.

50ul of freshly made solution B was gently mixed in. After 30sec 250ul of solution C was added and mixed carefully with a slow rotary motion.

The material was incubated at 10°C until it was clear. This took ~5min.

200ul of nucleoid material was added to a 10-30% (w/v) sucrose gradient and centrifuged in the TLS.55 rotor (Beckman) at 17,000g for 10min. This separated the nucleoids from the cellular debris which was found at the bottom of the tube and the cytoplasmic material which was retained at the top of the tube.

Solution A

10mM EPPS pH8.2, 10mM Sodium Azide, 0.1M NaCl, and 20% (w/v) sucrose.

Solution B

0.12M EPPS pH8.2, 50mM EDTA pH8.0, and 4mg/ml lysozyme.

Solution C

1% Brig-58 (Sigma), 0.4% Sodium Deoxycholate (Sigma), 2M NaCl, 10mM EDTA-Na_2 pH8.0.

Sucrose gradient solutions

10, 20, or 30% (w/v) sucrose, 10mM EPPS pH8.2, 1M NaCl, 1mM EDTA, and 1mM mercaptoethanol.

2.13.2 Lysis protocols for attempted nucleoid isolation from *Sulfolobus solfataricus* and AN1

Sulfolobus solfataricus was grown in the recommended media (DSM) at 75°C. Cells were adjusted to pH6.5 with 2M Tris before harvesting and then centrifuged at 8000g for 20min at 10°C. AN1 was grown as previously described (section 2.5). After inoculation and growth for 20hr the culture was put through a glass sinter filter to remove sulphur particles and the cells centrifuged at 8000g for 20min. *S. solfataricus* or AN1 cells were further processed in one of the following ways:

(a) Low salt lysis (from Reddy & Suryanarayana, 1988). 0.2g cells were resuspended in 0.5ml of 10mM Tris/HCl pH7.5 and 100mM NaCl. An equal volume of lysis solution consisting of 1% Nonidet P40 (BDH), 2mM spermidine, 10mM Tris/HCl pH7.5, and 10mM EDTA was added with gentle mixing. Lysis at various temperatures and times followed.

(b) High salt lysis (modified from Worcel & Burgi, 1972). 0.2g cells were resuspended in 0.5ml of 10mM Tris/HCl pH7.5 and 100mM NaCl. An equal volume of lysis solution consisting of 1% Bridj-58 (Sigma), 0.4% deoxycholic acid (sodium salt), 1M NaCl, 10mM EDTA (disodium salt) and 10mM Tris/HCl pH7.5, was added with gentle mixing. Lysis at various temperatures and times followed.

After lysis the solution was generally centrifuged at 1500g for 3min to precipitate intact cells and cell wall material. The supernatant was removed and used to determine the presence of nucleoids.

When running 10-30% (w/v) sucrose gradients of the lysed material, run times and speeds were varied in order to optimize banding of the postulated nucleoid fraction. In all cases acceleration and deceleration rates were set on 2. When using low salt lysis procedures the sucrose solutions consisted of 10, 20, or 30% sucrose, 10mM Tris/HCl pH7.5, 5mM $MgCl_2$, 1mM EDTA, and 1mM mercaptoethanol. When using high salt lysis procedures the sucrose solutions consisted of 10, 20, or 30% sucrose, 10mM Tris/HCl pH7.5, 1M NaCl, 1mM EDTA, and 1mM

mercaptoethanol.

2.13.3 Phase contrast microscopy

Phase contrast photomicrograph's of *E. coli* and *S. solfataricus*, cells, protoplasts, and nucleoids were taken on a Reichert-Jung Polyvar microscope (type 300602) using Ilford FP4-135 film. Samples were suspended in 50% glycerol to impede cell movement and photographs taken with a blue filter at a magnification of 1250.

2.13.4 Sucrose gradients and fraction collection

Sucrose density gradient centrifugation for isolation of nucleoids was performed in a TL100 benchtop ultracentrifuge using a TLS.55 swinging bucket rotor (Beckman Instruments). Linear 10-30% (w/v) sucrose gradients were formed in 11x34mm (2.2ml) polyallomer tubes (Beckman) by diffusion of 0.63ml layers of 30, 20, and 10% (w/v) sucrose for 1hr at 20-25°C. The procedure is outlined in application note 1 (Bioresearch DS.640, 1984) for the TL100 ultracentrifuge. Reproducible linear gradients (figure 2.13.1) were formed by pipetting 630ul of the 30% (w/v) sucrose solution into the tube and capping the tube with a plastic cap. A 1ml syringe with a 1.5 inch, 22 gauge needle was filled with 630ul of 20% (w/v) sucrose solution and the needle inserted through the plastic cap to the side of the tube just above the level of the 30% (w/v) sucrose solution. The rubber plunger was removed and the solution allowed to flow into the tube (figure 2.13.2). This procedure was repeated for the 10% (w/v) sucrose solution and the boundaries between the layers thus formed were clearly visible. The reproducibility of this method was determined by making 8 gradients and determining the final volume of each. The maximum variation in volume was 30ul (1.5%). After 1hr at 20-25°C the sucrose gradients were transferred to a 4°C cold room and left for 30min-1hr before use. After centrifugation fractions were collected by placing the tube into a home made tube guide (figure 2.13.3). A rubber Hungate lid was placed onto the centrifuge tube opening and screwed down onto the centrifuge tube to form an air tight seal.

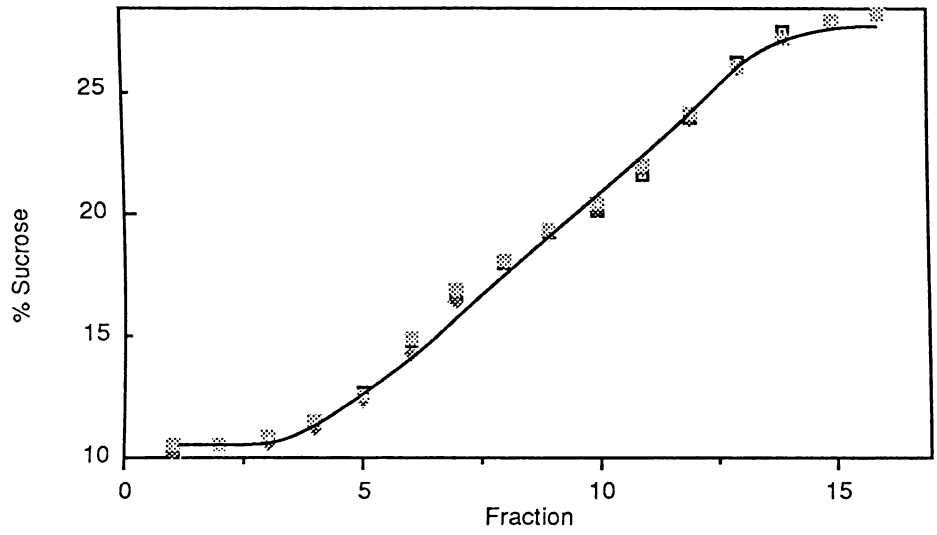


Figure 2.13.1 Graph showing results from the preparation of linear sucrose gradients and fraction collection. This experiment was conducted in triplicate and each fraction shows the data points from 3 gradients. The % sucrose in each fraction was determined using an Abbe refractometer (ATAGO, Japan).

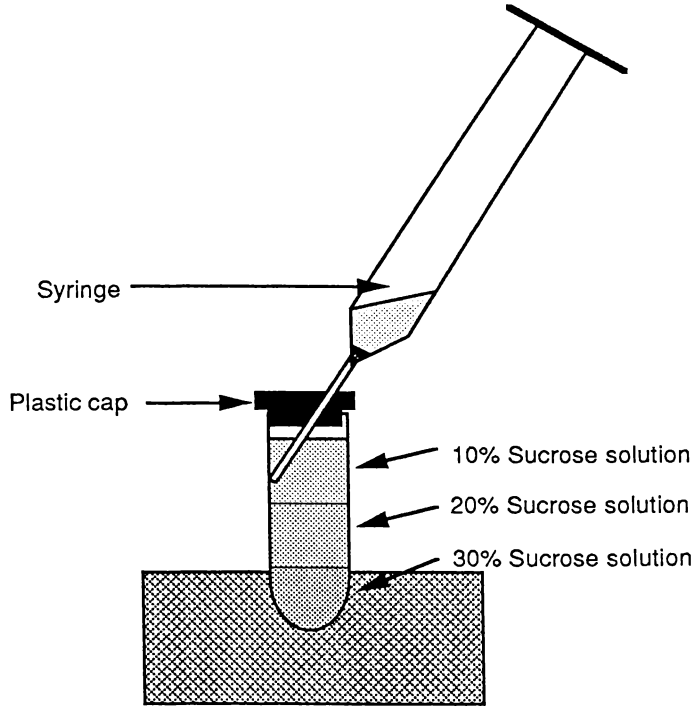


Figure 2.13.2 The preparation of 10-30% linear sucrose gradients using the layering technique.

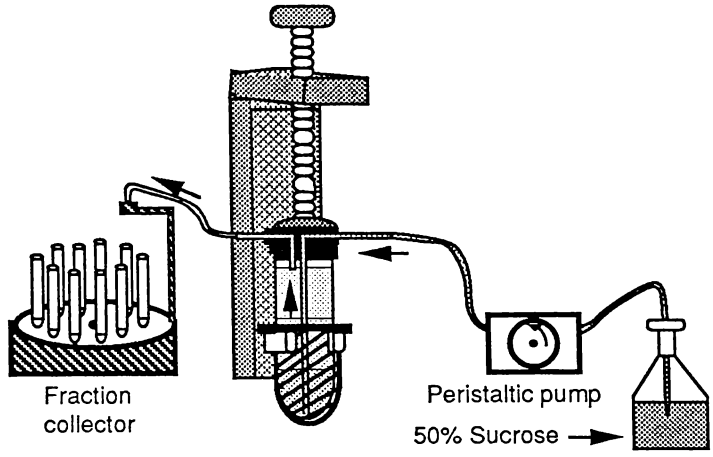


Figure 2.13.3 Fraction collection of sucrose gradients. The diagram is not to scale as the tube containing the sucrose gradient and the accompanying apparatus are magnified to show the details of the system.

Inserted into the rubber lid was a 21 gauge needle which extended down to the bottom of the centrifuge tube and a small bore (0.2mm) plastic tube which extended 2mm through the rubber lid. The needle was connected via plastic tubing to a peristaltic pump which pumped 50% (w/v) sucrose (15ml/hr) into the centrifuge tube. The contents of the centrifuge tube were thus forced through the small bore tube at the top of the centrifuge and onto a LKB fraction collector set to measure drops per fraction (figure 2.13.3). This procedure was found to be more reliable and accurate in collecting fractions then by collecting drops by a hole punctured at the bottom of the tube. Band diffusion during collection of material from the gradient was also minimized by this procedure.

2.13.5 Agarose gel and ethidium bromide fluorescence assays

Fractions taken from sucrose gradients were assayed for their nucleic acid content either by running on an agarose gel or by ethidium bromide fluorescence. The agarose gel procedure consisted of collecting fractions in 1.5ml eppendorf tubes adding an equal volume of phenol (prepared as described by Maniatis *et al.*, 1982) then mixing and centrifuging the samples at 5000g for 3min. 10ul of the top phase was removed and added to 2ul of SDS loading buffer. Gels were made and run as described in section 2.4. The procedure for using ethidium bromide fluorescence to detect nucleic acids involved collection of the sucrose gradient fractions into glass tubes followed by addition of 2ml of ethidium bromide fluorescence solution (0.5ug/ml ethidium bromide, 5mM Tris/HCl pH8.0, and 0.5mM EDTA). Fluorescence was then detected on

a Shimadzu digital spectrofluorophotometer RF-510 with an excitation wavelength set at 525nm, emission wavelength set at 600nm, excitation slit width set at 20nm, and the emission slit width set at 40nm (Morgan *et al.*, 1979).

2.13.6 Determination of the superhelical density of the *E. coli* nucleoid from ethidium bromide/sucrose density gradients

After production of nucleoids or nucleoid type structures 200ul of material was layered onto a sucrose gradient. From this preparative gradient 15 fractions were taken and the fraction of peak DNA content determined by spectrofluorometry. The peak fraction was diluted 1 in 3 with sucrose gradient buffer containing no sucrose to ensure that the material remained at the top of subsequent sucrose gradients as it was being layered on. This was used in the next series of sucrose gradients containing increasing concentrations of ethidium bromide or netropsin. The use of preparative sucrose gradients enabled the separation of heterogeneous nucleoid sizes and so produced tighter nucleoid bands in subsequent experiments (Worcel and Bergi, 1972). This gave a better resolution of changes in mobility of nucleoids due to ethidium bromide.

70ul of nucleoid material was layered onto sucrose gradients with different concentrations of ethidium bromide (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 7.0, 10.0ug/ml ethidium bromide) or netropsin (0, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 7.0, 10.0uM netropsin). In the case of *E. coli* nucleoid runs, T4 phage was used as an internal marker, however for runs with *S. solfataricus*, T4 was not added as it remained at the top of the gradient in the shorter run times employed for these structures. Centrifuge run conditions for *E. coli* nucleoids were 8600g for 10min and for *S. solfataricus* and AN1 nucleoid type structures run conditions were varied from 2700g for 12min to 6600g for 10min. Typically 38 to 40 fractions were taken from sucrose gradients and assayed for DNA content.

The superhelical density (σ) of DNA was defined as the number of superhelical turns per 10 base pairs and is determined by equation 1 (Bauer and Vinograd, 1968).

$$\sigma = \frac{10\,\phi\,r}{180°}$$

ϕ is the angle by which the DNA molecule is wound after intercalation of 1 molecule of drug. For ethidium bromide this is taken to be -26° (Liu and Wang, 1975). r is the ratio of bound

ethidium bromide per nucleotide and is determined by equation 2 (Scatchard, 1949).

$$r = \frac{V_m K C}{1 + K C}$$

V_m is the maximum number of binding sites of bound dye per nucleotide. A value of 0.2 was assumed for ethidium bromide (Le Pecq and Paoletti, 1967). K is the association constant which varies according to the ionic strength and temperature of the solution. At 4°C and 1M NaCl, K = 3.1 x 10^5l/M (Le Pecq and Paoletti, 1967). C is the free dye concentration in moles per litre. This is deduced by determining the concentration of dye (in this case ethidium bromide) which is necessary to effect total relaxation of the supercoiled DNA molecules.

2.13.7 Electron microscopy of nucleoid and protoplast structures

Protoplast and nucleoid samples were prepared for electron microscopy using the cytochrome monolayer technique essentially as described by Delius and Worcel, 1974 and Davis *et al.*, 1971.

A 10ul portion of nucleoid material from a sucrose gradient was carefully mixed, by slow rotation, with 50ul of the spreading solution containing a mixture of 33% (v/v) formamide, 22% 0.5M Tris/HCl pH8.5, and 2% Cytochrome C (0.5%). The sample was spread on a hypophase containing 20% formamide and 10mM Tris/HCl adjusted to pH8.7. This was made 5min or less before using as the pH of this solution dropped quickly. The delicate step of spreading the solution onto the hypophase solution was achieved as follows:

A chromic acid washed and milliQ H_20 rinsed petri dish (60x15mm) was filled with 15ml of hypophase solution. A cleaned glass slide was placed into the petri dish and held at an angle of 25° by a glass rod. 50ul of the DNA containing solution was slowly spread across the slide near the slide hypophase solution boundary. The film thus produced was allowed to stand 1min and then a parlodium coated grid (50-200 mesh) was gently and briefly touched onto the surface near the slide hypophase solution boundary.

Staining of the grids was achieved by gently draining the grid on tissue paper then dipping the grid for 30sec into 10^{-5}M uranyl acetate in 90% ethanol. The grid was rinsed for 10sec in 90% ethanol and allowed to dry.

Grids were rotary shadowed in a rotary shadower by placing them on a rotating table

(100rpm) and shadowed under vacuum using 30mg of platinum wire wound around a tungsten filament. The platinum was then evaporated onto the sample at a shadowing angle of 7° and a filament to specimen distance of 18cm.

CHAPTER THREE

THE DISTRIBUTION OF REVERSE GYRASE IN REPRESENTATIVE SPECIES OF EUBACTERIA AND ARCHAEBACTERIA

3.1 Introduction

Bacteria were grown, lysed and assayed for reverse gyrase activity as previously described (See section 2.5). Under the conditions of assay reverse gyrase activity was detected by the formation of positively supercoiled topoisomers from negatively supercoiled pBR322 (Figure 3.1.1 and 3.1.2). With the use of crude cell extracts the result obtained reflects the balance of all enzymes active on pBR322 as a substrate and as such must be interpreted with caution. However both prokaryotic and eukaryotic topoisomerases can be assayed in crude cell extracts (DePew *et al.*, 1978; Fairfield *et al.*, 1979; Thrash *et al.*, 1984; Roca and Mezquita, 1989; and Gellert *et al.*, 1976). It was therefore considered that the use of crude cell extracts was acceptable for general screening of topoisomerases provided that nuclease activity (which was in some cases discovered under the assay conditions used) was not predominant.

3.2 Results of screening for reverse gyrase activity

The distribution of reverse gyrase, along with other noted activities, in representative archaebacteria and eubacteria is summarised in table 3.2.1. Reverse gyrase is specific to the thermophilic sulphur metabolizing archaebacteria (figure 3.2.1). No positive supercoiling activity was detected in eubacteria or other archaebacteria (figure 3.2.2).

The phylogenetically unique genus *Thermoplasma*, shows no sign of reverse gyrase activity. This supports recent proposals which show that this genus has a closer relationship with the methanogens than with the sulphur metabolizing archaebacteria (Woese and Olsen, 1986).

Table 3.2.1 The distribution of reverse gyrase activity in representativespecies of eubacteria and archaebacteria.

Species	Strain number	Optimum growth temperature	Reverse gyrase present
Pseudomonas fluorescens	ATCC 13525	30°C	-
Escherichia. coli[1]	HB 101	37°C	-
Clostridium thermoaceticum	DSM 521	55°C	-
Clostridium thermohydrosulfuricum[1]	DSM 570	60°C	-
Thermobacteroides acetoethylicus[1]	ATCC 33265	65°C	-
Bacillus stearothermophilus[2]	DSM 22	55°C	-
Bacillus acidocaldarius	ATCC 27009	60°C	-
Bacillus caldovelox[2]	DSM 411	70°C	-
Bacillus caldolyticus[2]	DSM 405	70°C	-
Bacillus caldotenax[2]	DSM 406	70°C	-
Thermus thermophilus[1]	ATCC 27009	60°C	-
Thermus sp	ATCC 31674	75°C	-
Thermotoga sp[1]	TRUCC FjSS3-B.1	80°C	-
Halobacterium halobium[*]	DSM 670	37°C	-
Halobacterium saccharovorum[*]	ATCC 29252	37°C	-
Methanosarcina barkeri[*]	unknown strain	37°C	-
Methanobacterium thermoautotrophicum[*]	ATCC 29096	65°C	-
Methanococcus thermolithotrophicus[*]	DSM 2095	65°C	-
Thermoplasma acidophilum[*1]	ATCC 25905	55°C	-
Sulfolobus acidocaldarius[*]	FERM P.7137	75°C	+
Sulfolobus acidocaldarius[*]	ATCC 33909	75°C	+
Sulfolobus sp[*]	TRUCC RT.70	75°C	+
Sulfolobus sp[*]	TRUCC WI	75°C	+
Sulfolobus solfataricus[*]	DSM 1616	80°C	+
Desulfurococcus mobilis[*]	DSM 2161	85°C	+
Desulfurococcus mucosus[*]	DSM 2162	85°C	+
Desulfurococcus sp[*]	TRUCC TOK 12S.1	85°C	+
Desulfurococcus sp[*]	TRUCC RT 59S.1	85°C	+
Desulfurococcus sp[*]	TRUCC KET 55S.1	85°C	+
Thermoproteus tenax[*]	DSM 2078	85°C	+
Thermoproteus tenax[*]	DSM strain H_3	85°C	+
Thermoproteus tenax[*]	TRUCC TOK 12S.2	85°C	+
Thermococcus celar[*]	DSM 2476	85°C	+
Isolate AN1[*1]	TRUCC	75°C	+

* Denotes an archaebacterium. 1 Endonuclease activity detected in this bacterium.
2 Relaxing topoisomerse activity detected in this bacterium

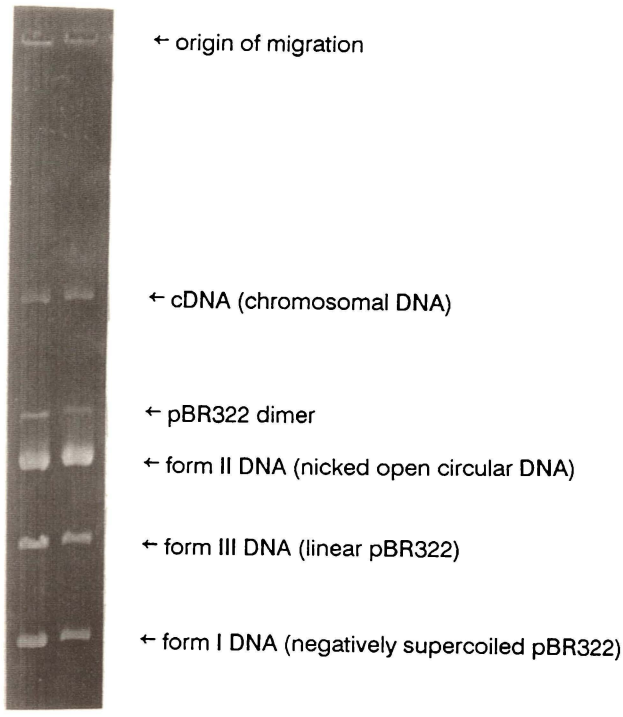


Figure 3.1.1 One dimensional gel of pBR322 run as described in section 2.4.

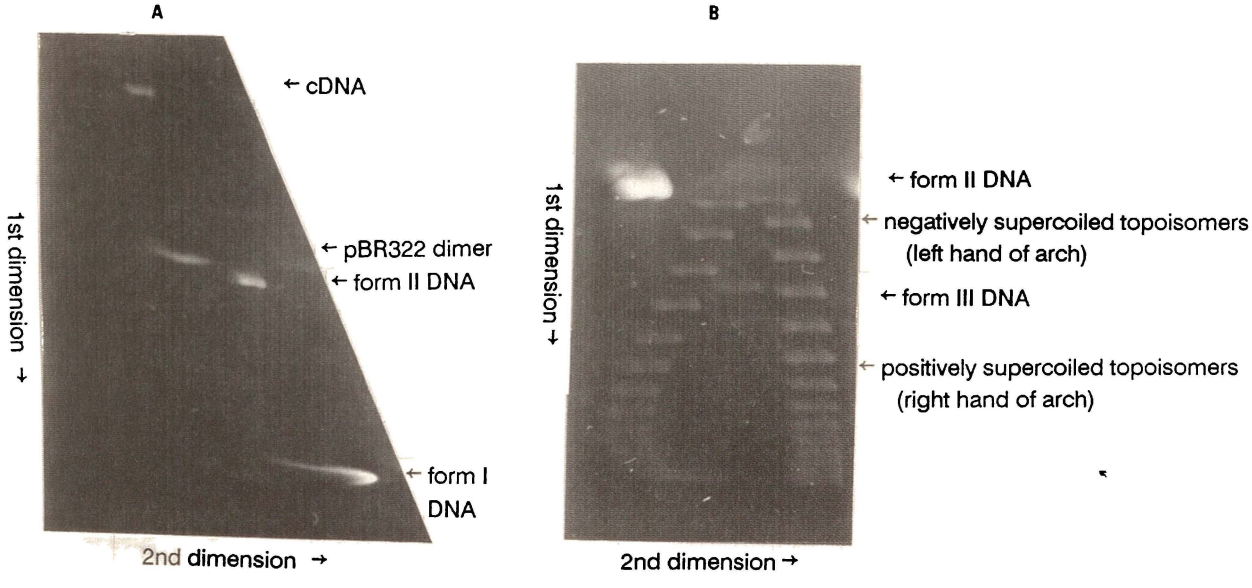


Figure 3.1.2 2D gels of:
(A) pBR322
(B) pBR322 incubated with reverse gyrase from *Thermoproteus tenax*
The conditions for running 2D gels are given in section 2.4.

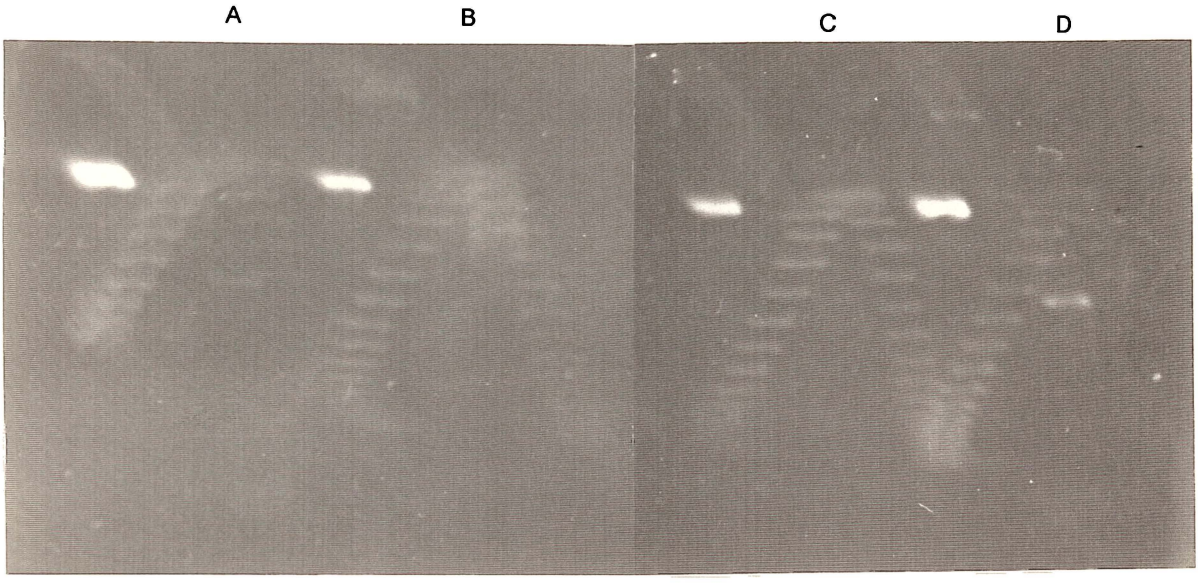


Figure 3.2.1 2D gels showing reverse gyrase activity from:
(A) *Thermoproteus tenax* Kra 1
(B) *Thermococcus celer*
(C) *Sulfolobus acidocaldarius*
(D) *Desulfurococcus mucosus.*

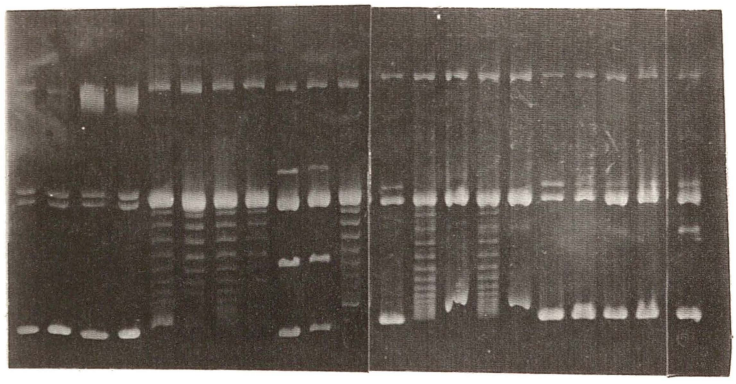


Figure 3.2.2 Screening of various bacteria for reverse gyrase activity. All crude extracts were assayed under standard conditions (see section 2.7) unless otherwise specified:
(a) & (b) *Halobacterium saccharovorum* incubated at 37°C for 1hr with (a) 0.5M KCl, and (b) 1.5M KCl in the assay mix
(c) & (d) *Halobacterium halobium* incubated at 37°C for 1hr with (c) 0.5M KCl,and (d) 1.5M KCl in the assay mix
(e) to (h) *Desulfurococcus mobilis* incubated at; 80°C for (e) 10min, and (f) 40min; 70°C for (g) 10min, and (h) 40min
(i) & (j) *Thermoplasma acidophilum* incubated for 40min at (i) 60°C, and (j) 75°C
(k) *Sulfolobus* sp strain RT70
(l) pBR3222 control (no enzyme) incubated at 75°C for 30min
(m) *Thermococcus celer* incubated at 75°C for 30min
(n) *Sulfolobus* sp White Island isolate incubated at 75°C for 30min.
(o) *Thermococcus celer* incubated at 75°C for 1hr
(p) *Sulfolobus* sp White Island isolate incubated at 75°C for 1hr
(q) & (r) *Pseudomonas fluorescens* incubated for 30min at (q) 37°C, and (r) 75°C
(s) & (t) *Methanosarcina barkeri* incubated for 30min at (s) 37°C, and (t) 75°C
(u) *Escherichia coli* incubated at 37°C for 30min.

In the sulphur metabolizing archaebacteria studied, AN1 excepted, reverse gyrase was the predominant activity noticed. Competing topoisomerase or nuclease activities in the crude extract were not detected and if present their activity was negligible under the assay conditions employed. It would appear that the predominant cellular topoisomerase is reverse gyrase (Jaxel *et al.*, 1989) and it is therefore likely that DNA in these cells is positively supercoiled.

3.3 Endonuclease activity in isolate AN1

The crude extract from isolate AN1 displayed both an endonuclease and a positively supercoiling activity (figure 3.3.1). The endonuclease is predominant in high concentrations of crude extract while the positive supercoiling activity predominates in lower concentrations of the crude extract. The endonuclease does not require ATP for activity and produces a partial digest of pBR322 with short incubation times (figure 3.3.2). The recognition sequence has been determined to be CTAG (K. Klages personal communication).

To ensure that the observed bands were not topoisomers, 2D gels of undiluted AN1 extract (figure 3.3.3) and lower concentrations of extract with and without ATP (figure 3.3.4) were run. In figure 3.3.3 the bands produced by the AN1 extract ran in a diagonal fashion on 2D gels. This is diagnostic of linear fragments whose mobility is not greatly affected by ethidium bromide in the second dimension. The mobility of the control topoisomers on the other hand is retarded by ethidium bromide. At lower concentrations of extract and with ATP present the positive supercoiling activity is predominant. In the absence of ATP a series of bands can be seen running in the diagonal manner characteristic of liner DNA fragments.

3.4 Endonuclease activity in other bacteria

Other bacteria displayed endonuclease activity during screening. These included *Thermus thermophilus, Clostridium thermohydrosulfuricum* and *Thermoanaerobacter acetoethylicus* (figure 3.4.1). Diagnostic of endonuclease activity but not topoisomerase activity is the disappearance of chromosomal DNA found in most preparations of pBR322. Nicking and linear cutting of the pBR322 molecule was also observed in some extracts, notably *Thermoplasma acidophilum, Thermotoga* sp, and *Escherichia coli*.

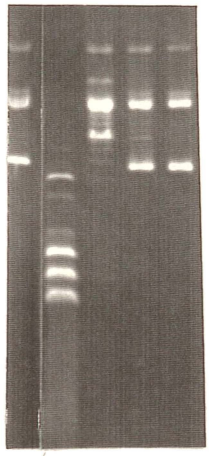


Figure 3.3.1 Activities from the crude extract of isolate AN1 under standard assay conditions:
(a) pBR322 (control)
(b) AN1 extract, undiluted
(c) AN1 extract, 10^{-1}
(d) AN1 extract, 10^{-2}
(e) AN1 extract, 10^{-3}.

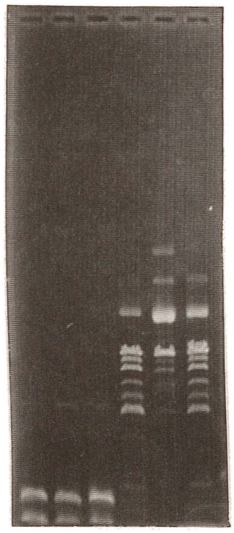
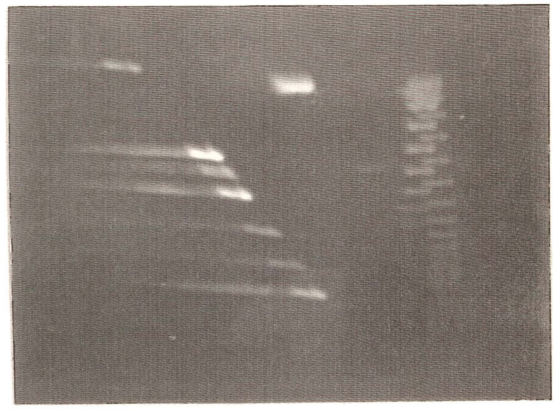


Figure 3.3.2 The effect of ATP and dGTP on the endonuclease activity of isolate AN1:
(a) & (b) & (c) Undiluted AN1 extract incubated at 75°C for 40min with (a) 0mM ATP, (b) 1mM ATP, and (c) 1mM dGTP
(d) & (e) & (f) Undiluted AN1 extract incubated at 75°C for 3min with (a) 0mM ATP, (b) 1mM ATP, and (c) 1mM dGTP.



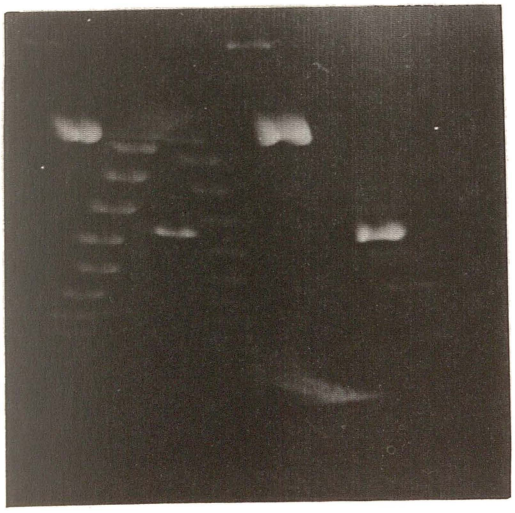


Figure 3.3.3 2D gel showing:
(a) pBR322 incubated with AN1 crude extract showing a diagonal ladder of cut linear DNA
(b) pBR322 incubated with *Sulfolobus* crude extract showing positive and negative topoisomers.

Figure 3.3.4 2D gel showing:
(a) AN1 extract (10^{-2}) with 1mM ATP
(b) AN1 extract (10^{-2}) without ATP.

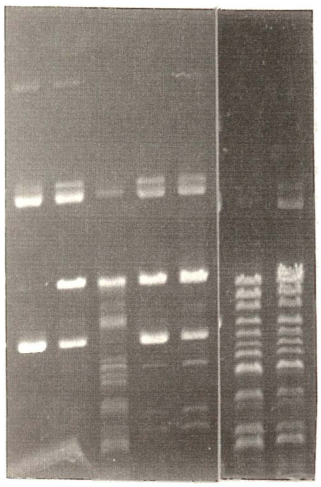


Figure 3.4.1 Endonuclease activities detected using pBR322 as a substrate:
(a) pBR322 (control)
(b) *Clostridium thermohydrosulfuricum* incubated at 60°C for 5min
(c) *Clostridium thermohydrosulfuricum* incubated at 60°C for 1hr
(d) *Thermobacteroides acetoethylicus* incubated at 60°C for 5min
(e) *Thermobacteroides acetoethylicus* incubated at 60°C for 1hr
(f) *Thermus thermophilus* incubated at 70°C for 10min
(g) *Thermus thermophilus* incubated at 70°C for 1hr.

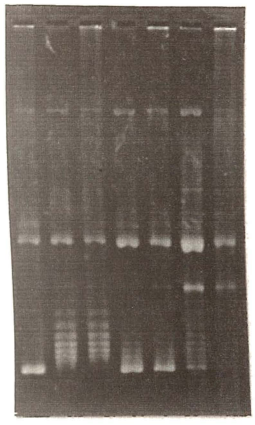


Figure 3.5.1 Topoisomerase activities from *Bacillus* strains:
(a) pBR322 (control)
(b) & (c) *Bacillus caldotenax* incubated at 70°C for (b) 10min, and (c) 1hr
(d) & (e) *Bacillus caldolyticus* incubated at 70°C for (d) 10min, and (e) 1hr
(f) & (g) *Bacillus stearothermophilus* incubated at 70°C for (f) 10min, and (g) 1hr.

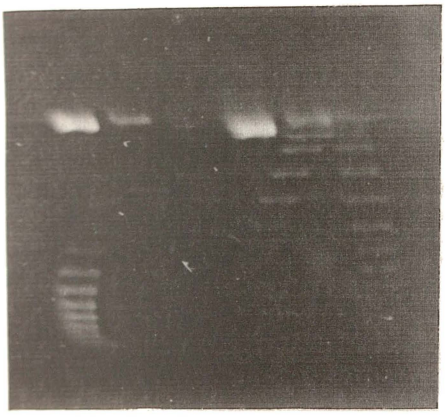


Figure 3.5.2 2D gels of topoisomerase activities from *Bacillus caldovelox* and a *Sulfolobus* strain:
(a) *Bacillus caldovelox*
(b) *Sulfolobus* sp.

3.5 Topoisomerase activity in other bacteria

Topoisomerase activities were noticed in *Bacillus caldotenax*, *Bacillus caldolyticus*, *Bacillus caldovelox* and *Bacillus stearothermophilus*. This activity did not require ATP and produced negatively supercoiled topoisomers only (figures 3.5.1 and 3.5.2).

The topoisomers produced by extracts of these thermophilic *Bacillus* species display a greater extent of negative supercoiling than seen in *E. coli* topoisomerase I. This is the result of the temperature of incubation at which these reactions are carried out. The centre of topoisomerase distribution is the ninth topoisomer from the nicked form II DNA. During cooling of the reaction mix from 75°C subsequent to loading onto a gel, thermally induced torsional fluctuations of the DNA molecules occur. A change in temperature from 75°C to 20°C has the effect of negatively supercoiling the pBR322 molecule by nine supercoils (based on a 4.3 Kb plasmid with a thermal unwinding angle of duplex DNA at $-1.4 \pm 0.1 \times 10^{-2}$/°C, base pair (Pulleybank *et al.*, 1975)). Therefore *Bacillus* topoisomerase is acting in a classical manner to completely relax the DNA substrate at the temperature of incubation.

CHAPTER FOUR

PURIFICATION AND CHARACTERIZATION OF REVERSE GYRASE FROM THE *THERMOCOCCUS* LIKE ISOLATE AN1.

4.1 Introduction

AN1 is a member of the *Thermococcales* branch of the archaebacterial kingdom (Klenk *et al.*, 1986). This group is phylogenetically distinct from other sulphur metabolizing archaebacteria to the extent that they may form a closer relationship with the methanogens and halophiles (Achenbach-Richter *et al.*, 1988). The possession of reverse gyrase by AN1 is thus interesting from a phylogenetic viewpoint and a comparison of its reverse gyrase with those found in the main sulphur metabolizing branch was expected to illuminate any differences that may exist between them.

Reverse gyrase has been purified from two archaebacteria to date, *Sulfolobus acidocaldarius* (Nadal *et al.*, 1988; Nakasu and Kikuchi, 1985) and *Desulfurococcus amylolyticus* (Slesarev, 1988). These two organisms show close phylogenetic relatedness despite the fact that *S. acidocaldarius* is an obligate aerobe while *D. amylolyticus* is obligately anaerobic. Reverse gyrase from both organisms has been characterized and no significant differences were noted except that the molecular weight of the enzyme apparently varies from 120 to 128kDa for *S. acidocaldarius* reverse gyrase (Nadal *et al.*, 1988; Nakasu and Kikuchi, 1985) to 135kDa for *D. amylolyticus* reverse gyrase (Slesarev, 1988).

The purification schemes utilized by Nakasu and Kikuchi, 1985, and Slesarev, 1988 were similar and comprised the following major steps:

a) Lysis of cells either by freezing and thawing (Nakasu and Kikuchi, 1985) or by ultrasonic disruption (Slesarev, 1988)
b) High speed centrifugation to clarify the supernatent
c) 0.3% polymin P to remove DNA
d) $(NH_4)_2SO_4$ precipitation
e) DEAE-Sephacel
f) Phosphocellulose (Whatman P11)
g) Heparin Sepharose

h) Sephacryl S-300 (Nakasu and Kikuchi, 1985) or refractionation on Heparin Sepharose (Slesarev, 1988).

Although Nakasu and Kikuchi, 1985 and Slesarev, 1988 did not mention any problems in their purifications regarding contaminating nuclease or protease activity these have been observed by Nadal *et al.*, 1988 and protease activity was observed during the course of this study. To overcome this problem Nadal *et al.*, 1988 developed an improved purification protocol for reverse gyrase which involved:

a) Cell lysis by homogenization
b) 0.36% Polymin P treatment at high ionic strength
c) High speed centrifugation
d) 35-70% $(NH_4)_2SO_4$ precipitation
e) Phenyl Sepharose
f) Phosphocellulose
g) Heparin Sepharose
h) 5-20% sucrose gradient fractionation

This protocol removed the effect of proteolytic action on reverse gyrase and completely separated nuclease activity from reverse gyrase at the Phenyl sepharose step. This purification procedure was unknown during the course of this study when reverse gyrase purification from AN1 was undertaken, however the important Phenyl Sepharose step from this procedure was used in a later *S. solfataricus* reverse gyrase purification (see Chapter 5). The final scheme developed to purify AN1 reverse gyrase was chosen using the following considerations: The use of gel filtration under high ionic conditions should preferentially separate the high molecular weight reverse gyrase from the majority of smaller proteins; trials with Blue Dextran Agarose had shown promise in the purification of reverse gyrase; and the newly introduced FPLC columns (Pharmacia), which combined improved reproducibility with convenience of use, were used in order to determine their effectiveness in reverse gyrase purification. Whatman P11 phosphocellulose prepared as described in the booklet "Use of Whatman Cellulose Phosphate Ion Exchanges, IL3" was not used in this purification scheme as in my hands, less than 10% of reverse gyrase activity bound to the column (3 attempts) under the starting conditions used by Nakasu and Kikuchi, 1985.

4.2 Purification of reverse gyrase from AN1

The procedures used to purify AN1 reverse gyrase are explained in detail in section 2.6. Reverse gyrase from 15gm of AN1 cells was purified 600 fold with a yield of 2.8% by the

purification scheme outlined in table 4.2.1 and elution profiles of purification steps from this scheme are shown in figures 4.2.1, 4.2.2, 4.2.3, 4.2.4, and 4.2.5. Reverse gyrase activity of pooled fractions between purification steps was quantified by determining the extent of relaxation of the negatively supercoiled substrate due to reverse gyrase (see section 2.3), 80% relaxation of negatively supercoiled pBR322 under standard assay conditions being equal to 1 unit of activity. Reverse gyrase activity was not quantified for individual fractions off a chromatographic run and the peak of and extent of activity was instead determined empirically by eye.

The activity of crude AN1 reverse gyrase could not be quantified after the high speed centrifugation step due to the high levels of endonuclease activity in the preparation. However activity was able to be quantified after $(NH_4)_2SO_4$ precipitation by serial dilution of the extract.

Fractogel TSK gel permeation chromatography of reverse gyrase was somewhat disappointing. Reverse gyrase activity eluted with the majority of the protein fraction and discrimination between proteins of differing molecular weight was not achieved to any great extent leading to a low degree of purification for reverse gyrase (figure 4.2.1).

During Fractogel TSK gel permeation chromatography the endonuclease activity co-eluted with reverse gyrase, however during Blue Dextran Sepharose chromatography, endonuclease activity did not bind to the column whereas reverse gyrase bound strongly and was not eluted from the column until a salt concentration of 0.8M NaCl was achieved. Of the protein applied to the column 94% did not bind and was washed from the column before the salt gradient was initiated. At 1M NaCl, the end of the linear gradient, reverse gyrase activity was still eluting from the column (figure 4.2.2). A column wash of 1.5M NaCl in 20mM Tris/HCl pH7.5 was therefore used to remove further activity. Active fractions from the linear salt gradient and the 1.5M NaCl, Tris/HCl pH7.5 wash were combined and used in the next purification step. The total activity found after purification on Blue Dextran Agarose was greater than that of the original solution. The reason for this is unclear but could well involve the removal of nucleases, or other inhibitors of the reverse gyrase assay, from the preparation at this stage. After ultrafiltration the sample was noticeably blue in colour, this was thought to be due to the elution of Blue Dextran not bound to the Agarose matrix. Upon loading the solution onto the MonoQ FPLC column the dye bound to the top of the column and was not removed by any of the conventional cleaning

procedures recommended for this column (MonoQ HR 5/5, Instructions, Pharmacia Fine Chemicals). Column performance however is apparently unaffected by such binding (Pharmacia sales representative, personal communication) though the use of Blue Dextran Agarose chromatography before FPLC chromatography was discontinued in subsequent purification procedures. During MonoQ FPLC chromatography 21% of the applied protein was washed from the column and 79% remained bound. Reverse gyrase was eluted from the column at 0.38M NaCl (figure 4.2.3).

The initial buffer conditions of MonoS FPLC chromatography were of critical importance as the pH and ionic conditions were required to be low in order for reverse gyrase to bind; however, the reverse gyrase solution from AN1 was insoluble at pH6.0 and OM NaCl. It was found that a MES/NaOH buffer at pH6.7 with 0.1M NaCl would satisfy both of these requirements and reverse gyrase eluted from the column during gradient formation at 0.4M NaCl (figure 4.2.4). At this step the majority of protein (84%) was not bound to the column. During the final Phenyl-Superose FPLC step the majority of protein (90%) applied to the column was again not bound; however reverse gyrase remained bound to the column and eluted as a single peak when a concentration of 0.25M $(NH_4)_2SO_4$ was reached on the linear gradient (figure 4.2.5). Active fractions were pooled, dialysed, and freeze dried. In order to determine the purity of the reverse gyrase fraction from Phenyl-Superose FPLC chromatography it was subjected to SDS-PAGE (10-20% gradient gel). One major band (120kDa) and one minor band (36kDa) were seen on the silver stained gel (figure 4.2.6).

Table 4.2.1 Purification of AN1 Reverse Gyrase. One unit of enzyme was defined as the amount of protein required for 80% relaxation of negatively supercoiled DNA under the standard assay conditions.

Purification step	Total protein (mg)	Total activity ($x10^4$ units)	Specific activity ($x10^3$ units/mg)	Yield (%)
Dialysed $(NH_4)_2SO_4$ pellet	442	47	1.1	100
Fractogel TSK gel permeation	127.5	46	3.6	98
Blue Dextran Agarose	9.3	61	67	129
MonoQ FPLC	5.5	40	73	85
MonoS FPLC	0.28	7.0	250	15
Phenyl-Superose FPLC	0.02	1.3	660	2.8

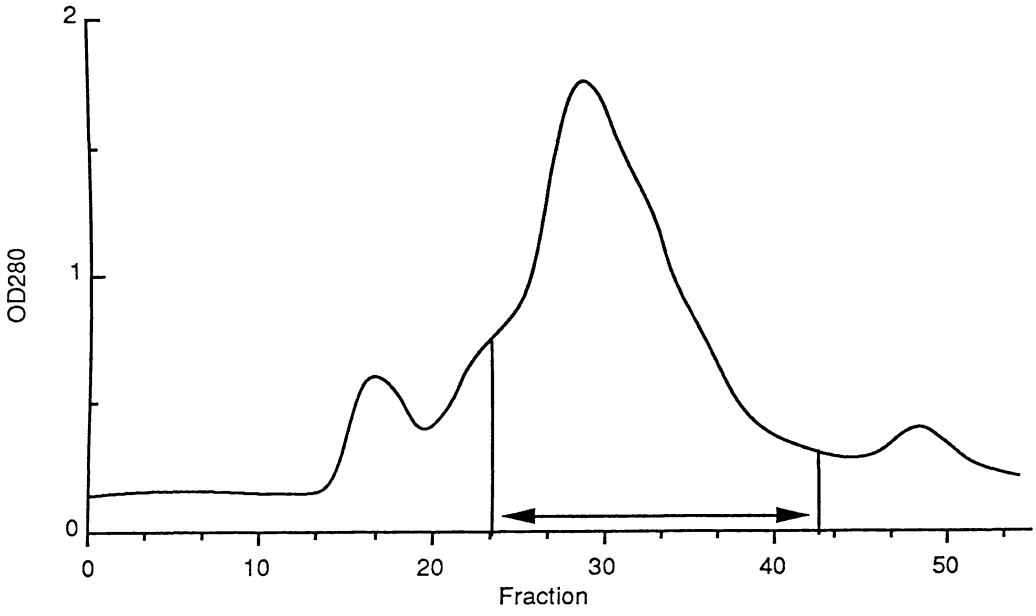


Figure 4.2.1 Gel permeation chromatography of AN1 reverse gyrase. Experimental details are described in section 2.6.3. Reverse gyrase activity was determined using the relaxation of cccDNA on agarose gels. Activity was not quantified when testing fractions from chromatographic steps and the peak and extent of activity was determined empirically by eye. The fractions with most activity, bounded by the vertical lines, were used for the Blue Dextran Chromatography step.

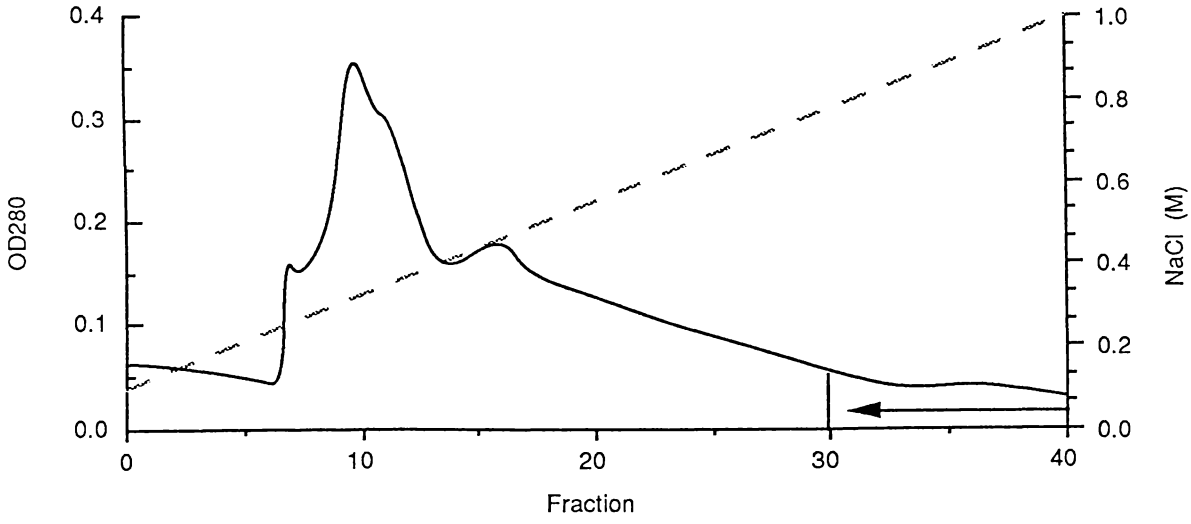


Figure 4.2.2 Blue Dextran Agarose chromatography of AN1 reverse gyrase. Experimental details are described in section 2.6.4. Reverse gyrase activity was determined using the relaxation of cccDNA on agarose gels and the most active fractions are shown bounded on the left by a vertical line. These fractions (30-40), and the fraction from the 1.5M NaCl wash, were used for the next step of anion exchange FPLC.

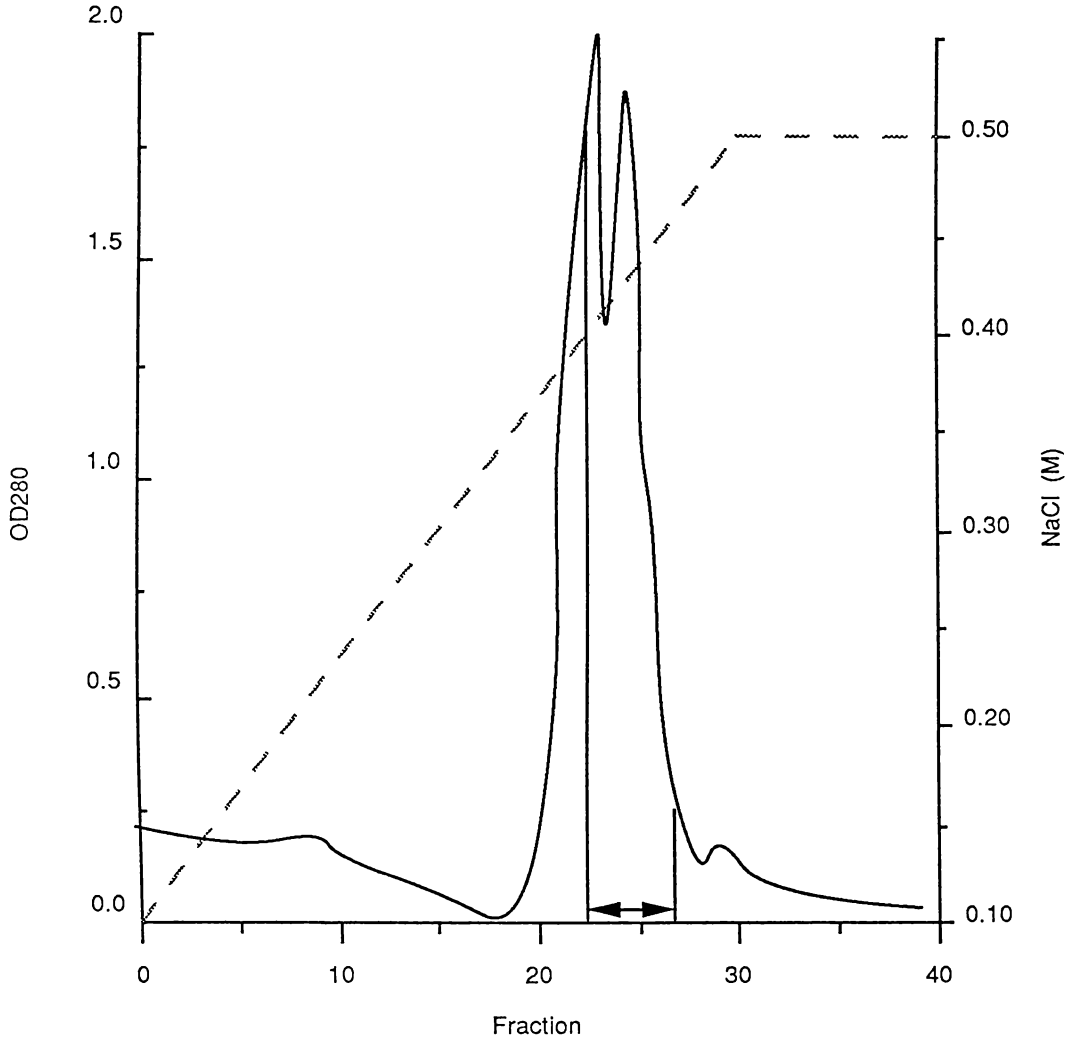


Figure 4.2.3 Anion exchange (MonoQ) FPLC of AN1 reverse gyrase. Experimental details are described in section 2.6.5. Reverse gyrase activity was determined in the standard manner. The fractions contained within the vertical lines displayed the majority of activity and were used in cation exchange FPLC.

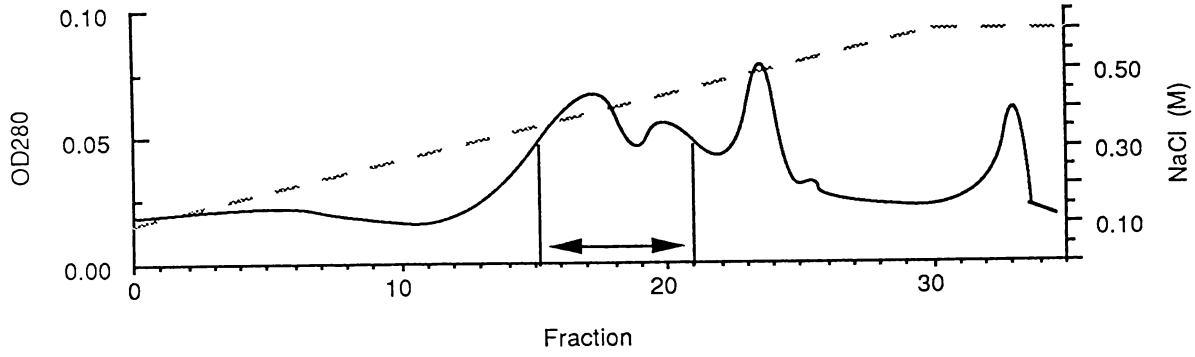


Figure 4.2.4 Cation exchange (MonoS) FPLC of AN1 reverse gyrase. Experimental details are described in section 2.6.6. The fractions bounded by the vertical lines contained the peak of activity and were used for Phenyl-Superose FPLC.

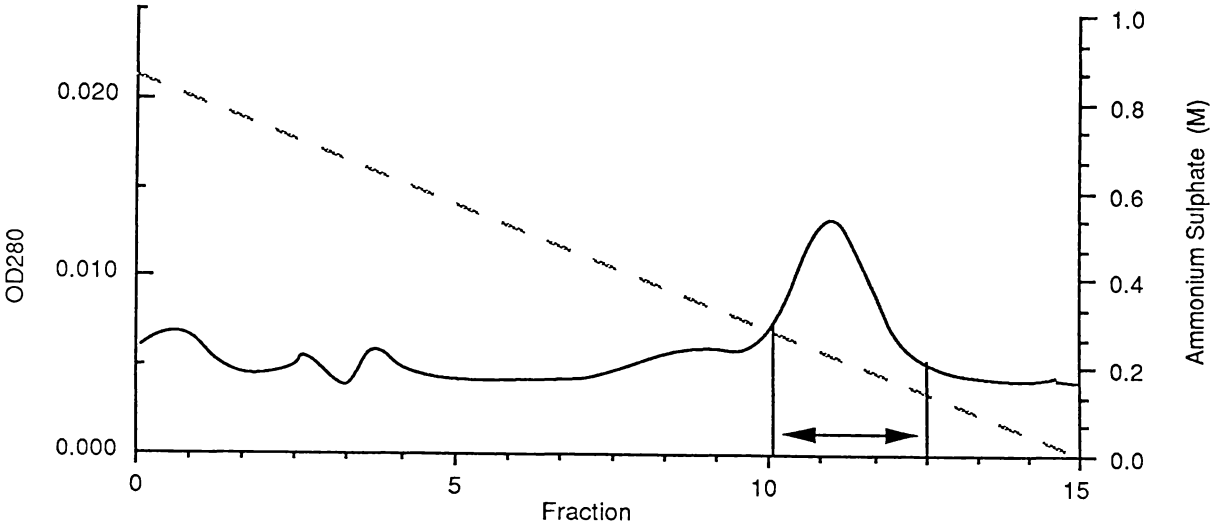


Figure 4.2.5 Hydrophobic interaction (Phenyl-Superose) FPLC. Experimental details are described in section 2.6.7. The peak of reverse gyrase activity coincided with the major protein peak and this fraction (bounded by vertical lines) was freeze dried after dialysis.

4.3 Characterization of reverse gyrase from AN1

The activity of reverse gyrase from AN1 was determined under different conditions by varying the parameter in question and keeping all other factors constant. The standard reverse gyrase assay was performed at 75°C for 10min and contained 50mM Tris/HCl pH7.5 (20°C), 0.2M NaCl, 10mM $MgCl_2$, 1mM spermidine, 1mM DTT, 1mM ATP, and 0.4ug negatively supercoiled pBR322 (Nakasu and Kikuchi, 1985).

4.3.1 Temperature

Activity of reverse gyrase was assayed under standard conditions with a 10min incubation at temperatures from 50 to 100°C. Initial experiments utilized a Tris/HCl buffer but this was discontinued due to the large temperature effect on pH which this buffer exhibits (ΔpKa/°C of -0.028, a pH change from 50-100°C of -1.4). 50mM MES/NaOH was substituted for Tris/HCl in later experiments as this buffer exhibited a smaller change in pH upon a temperature increment or decrement (ΔpKa/°C of -0.011, a pH change from 50-100°C of -0.55). Figures 4.3.1 (1D gel) and 4.3.2 (2D gel) show the results of temperature on AN1 reverse gyrase activity. Despite the rather blurred track at 95°C in figure 4.3.1, optimal activity appears to coincide with an assay

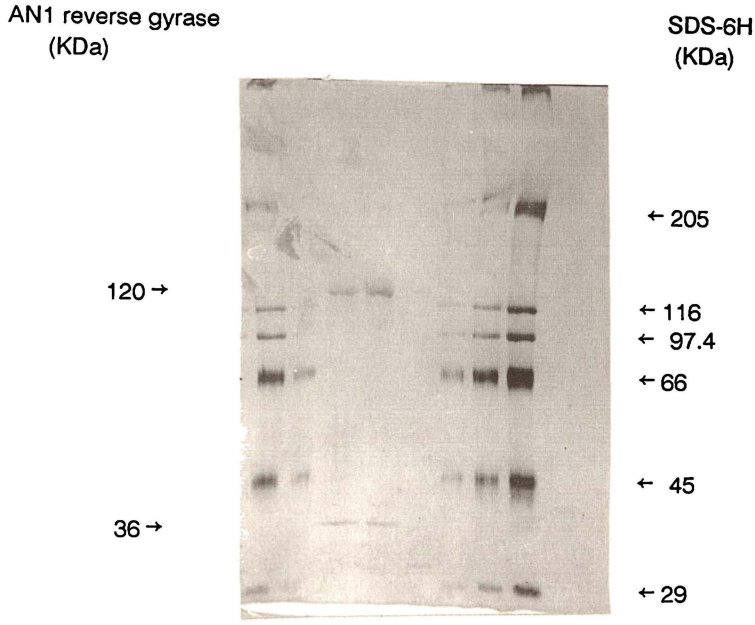


Figure 4.2.6 SDS-PAGE (10-20% polyacrylamide gradient) of purified AN1 reverse gyrase after hydrophobic interaction FPLC. Samples were prepared by heating at 100°C for 5min in Laemmli sample buffer. The resolving gel was 10-20% polyacrylamide and the stacking gel 4%. Electrophoresis was at 15°C with 13mA in the stacking gel and 18mA in the separating gel. Electrophoresis was completed after 6hr in the separating gel and the gel visualised by silver staining. Samples are: Lanes 1 & 6, SDS-6 molecular weight markers (Sigma) 1/40 dilution; Lanes 2 & 5, SDS-6 molecular weight markers (Sigma) 1/80 dilution; Lane 7, SDS-6 molecular weight markers (Sigma) 1/20 dilution; Lanes 3 & 4, purified AN1 reverse gyrase with 20 and 40ul of material repectively.

temperature of 90°C and this result is also seen upon two dimensional gel electrophoresis of the samples (figure 4.3.2).

The temperature 'optimum' of this or any enzyme is the result of a number of factors including, the ionic composition of the buffer, the presence of contaminating proteins, and the time taken to assay for activity. However two main factors are involved in determining the temperature at which peak activity occurs; firstly, an increase in the catalytic activity of the enzyme as temperature increases, and second, the progressive denaturation of the enzyme as temperature increases. For a given assay period the peak of activity is found at a temperature where the effects of these two factors cancel each other so that an increase in temperature denatures the enzyme to an extent which reduces total activity while a decrease in temperature decreases the activity of the enzyme to reduce total activity.

pH differences at the different temperatures assayed were confined to 0.33 and such a change was unlikely to be a significant factor in influencing the activity of reverse gyrase in these experiments.

Taking these and other results into account it was concluded that the optimum temperature for AN1 reverse gyrase under the assay conditions employed was 85-90°C. This makes reverse gyrase the most thermophilic topoisomerase reported (see table 4.4.1).

4.3.2 pH

Reverse gyrase activity under conditions of differing pH was determined under standard reverse gyrase assay conditions at a temperature of 90°C for 10min. In order to achieve a wide pH range three different buffer systems,at 50mM concentrations, were used; Na Acetate/Acetic Acid for a pH range of 4.0-5.5, MES/NaOH for a pH range of 4.6-5.9, and MOPS/NaOH for a pH range of 6.2-7.4. Each buffer was adjusted to compensate for pH variations due to temperature and dilution; for example, in order to produce a pH of 6.5 at 90°C with a MOPS/NaOH buffer ($\Delta pKa/°C = -0.006$) the pH at 20°C must be 6.92. A further pH adjustment of 0.1 is necessary to take into account dilution effects from the 1M stock MOPS/NaOH solution to the final 50mM buffer. In order to achieve a final pH of 6.5 at 90°C with MOPS/NaOH a 1M stock solution at 20°C will therefore need to be pH7.02.

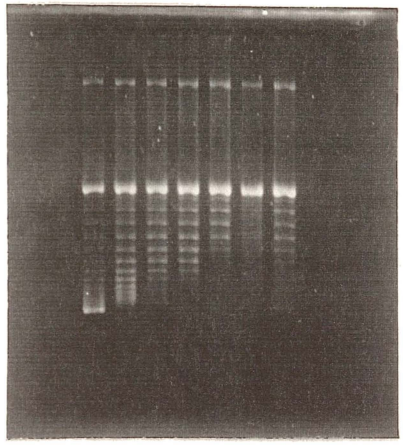


Figure 4.3.1 Effect of temperature on AN1 reverse gyrase activity (1D gel). Standard reverse gyrase assay conditions with 50mM MES/NaOH pH6.0-5.7 (70°C-100°C) as the buffer were used for these experiments. Lanes, from left to right, show reverse gyrase assayed at; 70, 75, 80, 85, 90, and 100°C.

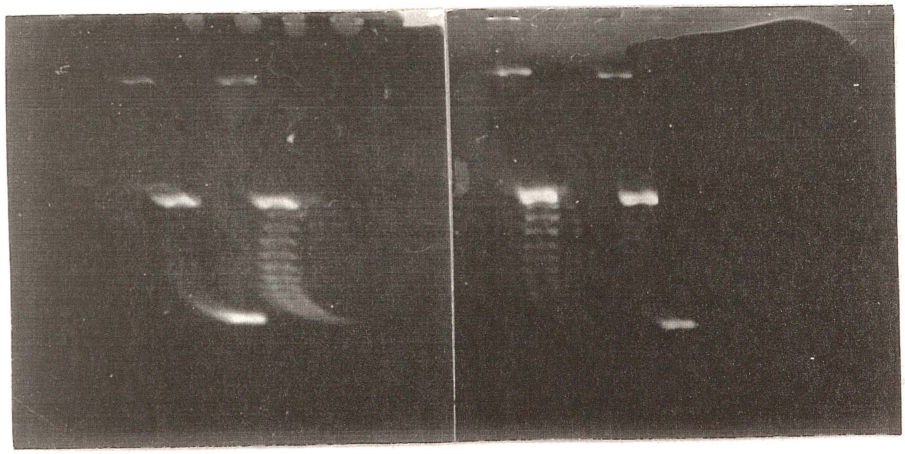


Figure 4.3.2 Effect of temperature on AN1 reverse gyrase activity (2D gel). Standard reverse gyrase assay conditions with 50mM MES/NaOH buffer were used for these experiments. Lanes, from left to right, were assayed at; 70, 80, 90, and 100°C.

Figures 4.3.3 (1D gel) and 4.3.4 (2D gel) show the effect of different pH's on reverse gyrase activity. From figure 4.3.3 it can be seen that reverse gyrase is active over a broad pH range of 5.5-7.0. Optimum activity of reverse gyrase within this range was determined to be at a pH of 6.3-6.5 by activities on two-dimensional gel electrophoresis, some of which are shown in figure 4.3.4.

4.3.3 Salt and magnesium requirements

The effect of NaCl on reverse gyrase activity was determined under standard assay conditions with 50mM MOPS/NaOH pH6.9(20°C) as the buffer and at a temperature of 85°C for 10min. A range of NaCl concentrations from 0-0.5M were examined, the results being shown in figures 4.3.5 (1D gel) and 4.3.6 (2D gel). From these results it was concluded that reverse gyrase displayed optimum activity in the range of 200-250mM NaCl while activity could be discerned over a wider range of 50-300mM NaCl. Under conditions of 200-250mM NaCl, AN1 reverse gyrase appears to act distributively and corresponds to the optimum activity of *S. acidocaldarius* reverse gyrase (160-200mM NaCl) determined by Nakasu and Kikuchi, 1985. Forterre *et al.*, 1985 reported that *S. acidocaldarius* under low salt concentrations (20-60mM KCl) acted in a fast processive manner with the direct appearance of positive supercoils, although a large part of the DNA substrate remained negatively supercoiled during short incubations. This processive activity was not determined for AN1 reverse gyrase and would not be evident on one dimensional gels unless a second gel containing ethidium bromide or chloroquine was also run to act as a comparison.

The effect of Mg^{2+} on reverse gyrase activity was determined as for the NaCl experiments and with 200mM NaCl. Concentrations ranging from 0-192mM $MgCl_2$ were tested and the results on reverse gyrase activity are shown in figures 4.3.7 (1D gel) and 4.3.8 (2D gel). Optimum activity under these assay conditions was found at a magnesium concentration of 6-12mM while activity could be discerned in a range from 0.5-24mM magnesium.

A requirement for magnesium has been found in all topoisomerases except eukaryotic topoisomerase I (table 4.4.1). Its function in eubacterial type I topoisomerases is uncertain although it appears not to be bound to the purified enzyme which does however contain 3 bound zinc atoms (Tse-Dinh and Beran-Steed, 1988). In type II topoisomerases one of the functions of

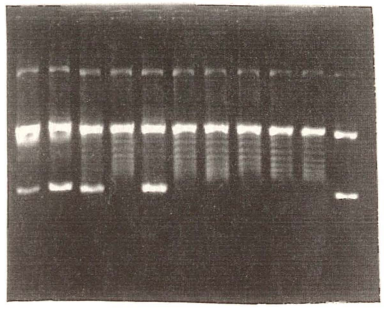


Figure 4.3.3 pH optimum of AN1 reverse gyrase (1D gel). Assays were performed at 90°C under standard assay conditions with the buffer (50mM) being changed to achieve the desired pH. The pH values used were those determined for the temperature at which the assay was performed (90°C). Lanes, from left to right, were assayed at; pH4.0 (NaAcetate/Acetic acid), pH4.5 (NaAcetate/Acetic acid), pH5.0 (NaAcetate/Acetic acid), pH5.5 (NaAcetate/Acetic acid), pH4.6 (MES/NaOH), pH5.4 (MES/NaOH), pH5.9 (MES/NaOH), pH6.2 (MOPS/NaOH), pH6.5 (MOPS/NaOH), pH7.0 (MOPS/NaOH), and pH7.5 (MOPS/NaOH).

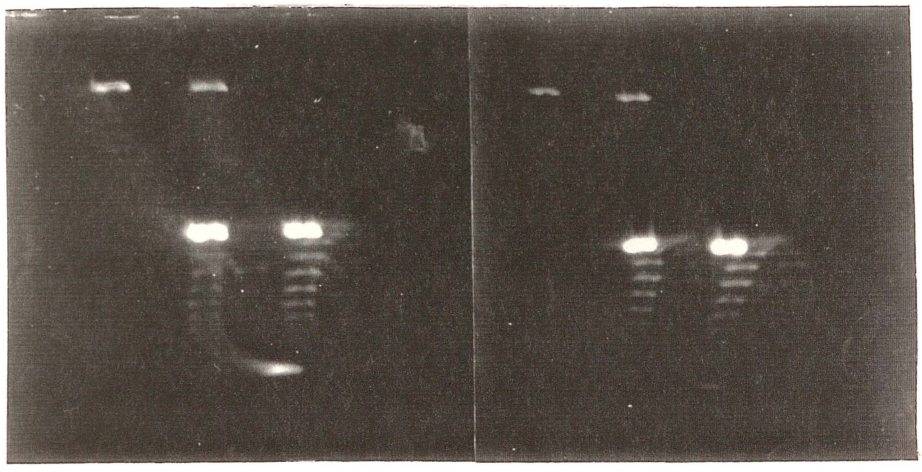


Figure 4.3.4 pH optimum of AN1 reverse gyrase (2D gel). Assays were performed as described in figure 4.3.3. In all cases 50mM MOPS/NaOH, adjusted for temperature effects, was used as the buffer. Lanes, from left to right, show assays performed at; pH5.5, pH6.3, pH6.1, and pH6.5.

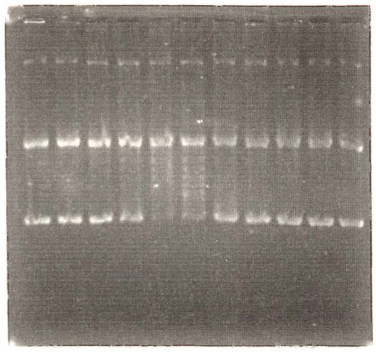


Figure 4.3.5 Optimal salt concentration for AN1 reverse gyrase (1D gel). Assays were performed under standard reverse gyrase assay conditions with 50mM MOPS/NaOH pH6.9 (20°C) as the buffer and at a temperature of 85°C for 10min. Lanes, from left to right, show AN1 reverse gyrase assayed with NaCl concentrations of; 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500mM NaCl.

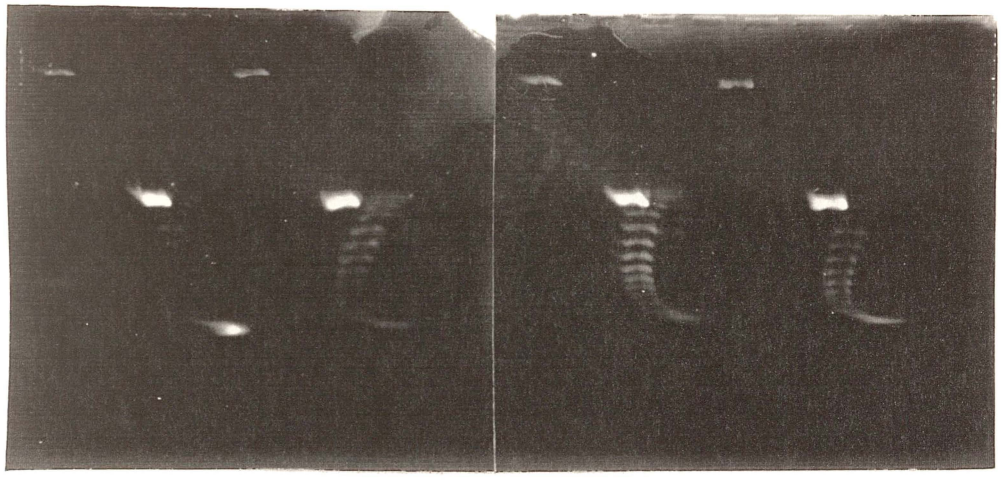


Figure 4.3.6 Optimal salt concentration for AN1 reverse gyrase (2D gel). Assay conditions are described in figure 4.3.5. Lanes, from left to right, show the effects of; 150, 200, 250, and 300mM NaCl on AN1 reverse gyrase activity.

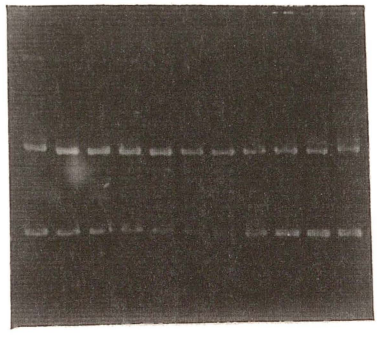


Figure 4.3.7 Optimal Mg^{2+} concentration for AN1 reverse gyrase (1D gel). Assay conditions are described in figure 4.3.5 with the addition of 200mM NaCl and with varying concentrations of magnesium. Lanes, from left to right, shows assays with; 0, 0.5, 1, 2, 3, 6, 12, 24, 48, 96, and 192mM $MgCl_2$.

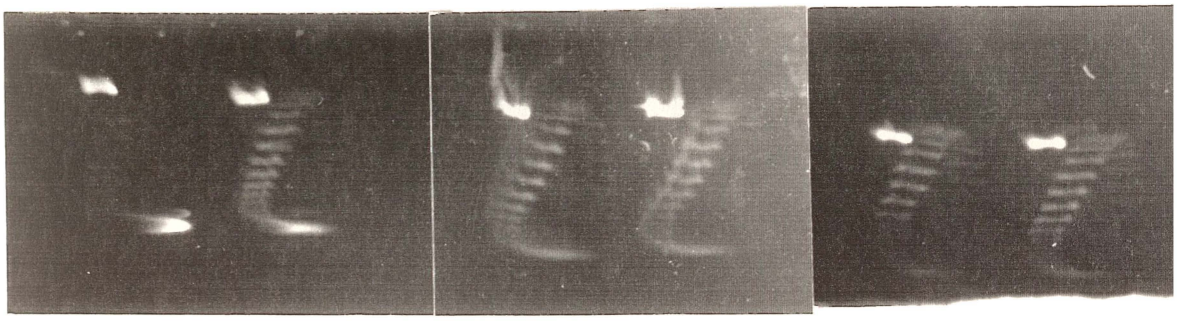


Figure 4.3.8 Optimal Mg^{2+} concentration for AN1 reverse gyrase (2D gel). Assay conditions are the same as those described in figure 4.3.7. Lanes, from left to right, show assays with; 0.5, 3, 6, 9, 12, and 24mM $MgCl_2$.

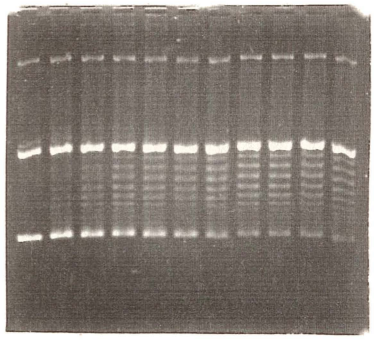


Figure 4.3.9 Effect of ATP on AN1 reverse gyrase activity (1D gel). The conditions of assay are the same as those described in figure 4.3.5 with the addition of 200mM NaCl and with varying concentrations of ATP. Lanes, from left to right, show reverse gyrase assayed with; 0, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024uM ATP.

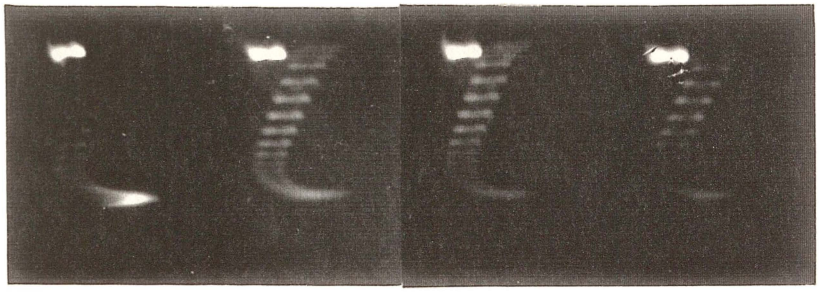


Figure 4.3.10 Effect of ATP on AN1 reverse gyrase activity (2D gel). The assay conditions are described in figure 4.3.9. Lanes, from left to right, show reverse gyrase activity assayed with; 2, 16, 512, and 256uM ATP.

magnesium is the formation of a magnesium ATP complex required for ATP hydrolysis (Osheroff, 1987). Magnesium may have a similar function in activating ATP for reverse gyrase. This is suggested from the observation that when ATP or Mg^{2+} is lacking during the reaction of reverse gyrase with cccDNA then nicked circles are formed (Nakasu and Kikuchi, 1985).

4.3.4 ATP

The effect of ATP on reverse gyrase activity was assayed as for the NaCl experiments with 200mM NaCl and 10mM $MgCl_2$. The effect of varying concentrations of ATP on reverse gyrase activity are shown in figures 4.3.9 (1D gel) and 4.3.10 (2D gel). From these results it was concluded that ATP is essential for activity and will support some activity even at very low concentrations (2uM) when compared to other ATP utilizing topoisomerases e.g *Sulfolobus* type II topoisomerase (>300uM for optimal activity) (Nakasu and Kikuchi, 1985). Full activity of reverse gyrase is supported by ATP concentrations greater than 128uM, if the disappearance of negative supercoils on 1D gels are considered (figure 4.3.9), or 500uM if the production of positive supercoils on 2D gels are considered (figure 4.3.10). 2D gels of reverse gyrase activity with ATP concentrations greater than 512uM (not shown) yielded the same number of positive supercoils as seen for the 512uM ATP assay. These figures are high in comparison to those found by Kikuchi *et al.*, 1986 who found that 10uM ATP was sufficient to completely transform negatively supercoiled DNA into a positively supercoiled form. Such a difference may well reflect a difference in enzyme concentration or assay conditions rather than an inherent difference between the two enzymes.

4.3.5 Thermostability of AN1 reverse gyrase

Due to a paucity of material at this stage of the characterization the thermostability of purified AN1 reverse gyrase was not thoroughly examined, however some preliminary trials were carried out. Purified AN1 reverse gyrase was diluted 50% in dilution buffer (50mM Tris/HCl pH7.5, 50ug/ml bovine serum albumin, 1mM spermidine) and incubated in 400ul capacity ependorf tubes at varying temperatures and for different times. The enzyme fractions were then assayed for activity at 80°C for 10min in an assay mixture containing; 50mM MOPS/NaOH pH6.9

(20°C), 200mM NaCl, 10mM $MgCl_2$, 1mM ATP, 1mM spermidine, 1mM DTT, and 0.4ug negatively supercoiled pBR322. The results of these experiments are shown in figure 4.3.11. It should be noted that figure 4.3.11 displays the results of only one experiment for each of the two temperatures tested. Further experiments were not carried out due to a lack of material but a second experiment at 85°C was run and gave results which differed somewhat from the first attempt. I consider that the reproducibility of this particular experimental procedure is in doubt although the results obtained should give a general indication of the thermostability of this enzyme. Denaturation of this enzyme apparently does not follow first order kinetics as the data points used in figure 4.3.11 do not form a straight line when the log of relative activity against time is plotted. The half lives for the enzyme at the two temperatures employed were found to be: 85°C $t_{½}$=14min, 90°C $t_{½}$=2.5min. These half lives are not likely to correspond to the half life of reverse gyrase *in vivo* where environmental conditions are markedly different from those used in the *in vitro* assay.

The measured half lives of reverse gyrase also incorporated the denaturation of the enzyme which occured during the assay for activity at 80°C for 10min. However the contribution which this made to the result was not ascertained.

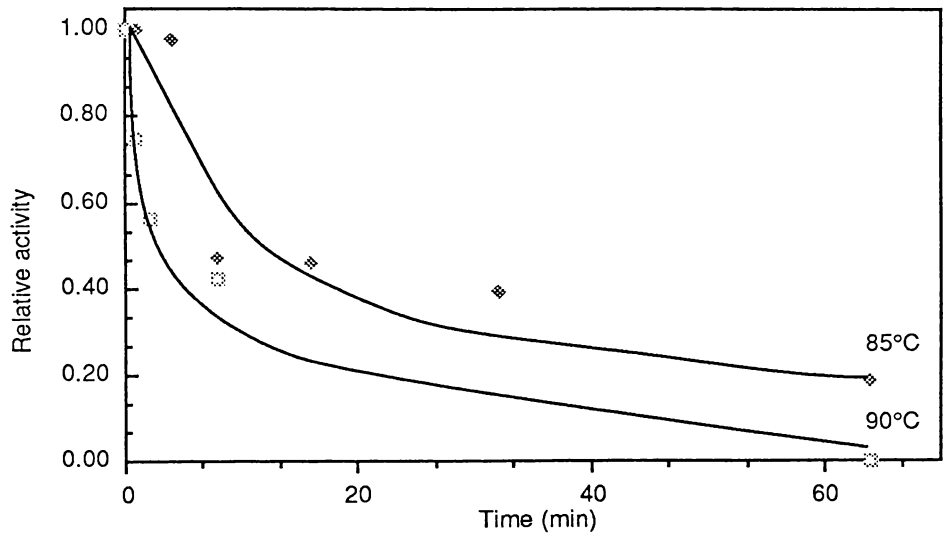


Figure 4.3.11 Thermostability of AN1 reverse gyrase. AN1 reverse gyrase was incubated for varying times at 85 and 90°C and then assayed at 80°C for 10min in; 50mM MOPS/NaOH pH6.9 (20°C), 200mM NaCl, 5mM $MgCl_2$, 1mM ATP, 1mM spermidine, 1mM DTT, and 0.4ug negatively supercoiled DNA.

4.3.6 The molecular weight of AN1 reverse gyrase

The molecular weight of purified AN1 reverse gyrase was determined using gradient SDS PAGE (10-20%) (figure 4.2.6). Upon silver staining two bands could be discerned, a major band at 120kDa and a minor band at 36kDa. These molecular weights were determined by a densitometric analysis of the gel using scanning laser densitometry and molecular weight analysis using Hoeffer GS-365 Data System software. It is proposed that AN1 reverse gyrase, whose activity corresponds to the major peak found in the final purification step of Phenyl-Superose FPLC chromatography, has a molecular weight of 120kDa. The 36kDa band is thought to be a contaminant and not a cleavage product of the 120kDa protein because proteolysis of the larger protein would generate a number of fragments and its molecular weight precludes it from being a trimer or tetramer of the 120kDa protein (120/36 = 3.3).

4.4 A comparison of AN1 reverse gyrase

The reverse gyrase from AN1 is similar to those described for *S. acidocaldarius* and *D. amylolyticus* and no real difference could be discerned between the three enzymes using the characteristics selected. The only possible differences occur in the area of:

(a) The temperature optimum for reverse gyrase which for AN1 was determined to be 85-90°C under the assay conditions used, for *D. amylolyticus* 100°C or greater (Slesarev, 1988), and for *S. acidocaldarius* 75-80°C (Kikuchi *et al.*, 1986). In all cases reverse gyrase was assayed for 10min. These differences may well reflect a difference in enzyme concentration or assay constituents e.g. ionic strength, rather than a real difference in temperature optima between the three enzymes.

(b) The molecular weight of reverse gyrase. The reported molecular weights for, *S. acidocaldarius* reverse gyrase are 120kDa (Nakasu and Kikuchi, 1985), 128kDa (Nadal *et al.*, 1988), and 132kDa (Forterre *et al.*, 1985), for *D. amylolyticus* reverse gyrase 135kDa (Slesarev, 1988), and for AN1 reverse gyrase 120kDa. These results are similar and until direct comparisons are carried out in the same laboratory I believe that it is speculative as to whether any real difference in the molecular weight of reverse gyrase exists between species or strains of archaebacteria with this enzyme.

Reverse gyrase is compared against the requirements of other topoisomerases for

activity in table 4.4.1. Topoisomerases are well known to be highly conserved enzymes, no doubt due to their essential functions in the cell and it appears that the reverse gyrase enzyme follows this trait.

Table 4.4.1 Optimum assay conditions for topoisomerases[a]

Source	Ionic Conditions[b]	Reference
Eubacterial Topoisomerase I[c]	0-40mM KCl 2-3mM Mg^{2+}	Champoux,1978 Depew *et al.*,1978
E. coli topoisomerase III	80mM KCl, 4mM Mg^{2+}	Srivenugopal *et al.*, 1984
Eubacterial DNA gyrase	25mM KCl, 7.5mM Mg^{2+} 2mM spermidine	Srivenugopal *et al.*, 1984
Eukaryotic topoisomerase I	100-200mM KCl 0mM Mg^{2+}	Champoux, 1978 Roca and Mezquita, 1989
Eukaryotic topoisomerase II	100-200mM KCl 10mM Mg^{2+}	Goto and Wang, 1982
Archaebacterial reverse gyrase	160-200mM KCl 3-10mM Mg^{2+} 1mM spermidine	Nakasu and Kikuchi, 1985

Source	Energy Requirement	Reference
Eubacterial topoisomerase I	no requirement	Andera and Mikulik, 1990
Eubacterial DNA gyrase	1.4-1.7mM ATP[d]	Gellert *et al.*, 1976 Bates and Maxwell, 1989
Eukaryotic topoisomerase I	no requirement	Thrash *et al.*, 1984
Eukaryotic topoisomerase II	0.2-1.0mM ATP	Goto and Wang, 1982 Roca and Mezquita, 1989
Archaebacterial reverse gyrase	10uM or greater	Nakasu and Kikuchi, 1985

Source	Temperature and pH requirements	References
Eubacterial topoisomerase I	Temp 37°C, pH7.5-8.5[e]	Andera and Mikulik, 1990
Bacillus stearothermophilus	Temp 65-70°C pH7.5-8.0	Yamamoto *et al.*, 1985
Eubacterial DNA gyrase	Temp 25°C, pH7.5	Gellert *et al.*, 1976
Eukaryotic topoisomerase I	Temp 20-37°C pH7.5-8.0	Thrash *et al.*, 1984
Eukaryotic topoisomerase II	Temp 30-37°C pH7.5	Goto and Wang, 1982 Roca and Mezquita, 1989
Archaebacterial reverse gyrase	75-100°C pH6.2-6.5	Slesarev, 1988 This thesis

[a] The four topoisomerase groups comprising; eubacterial topoisomerase I, eubacterial DNA gyrase, eukaryotic topoisomerase I, and eukaryotic topoisomerase II, appear to be well conserved within each group. All eukaryotic topoisomerase I's, for example, generally display optimum activity under the same conditions. The eubacterial type I topoisomerases from *E. coli* (topoisomerase III) and *B. stearothermophilus* are also included in the table.

[b] In most cases NaCl will substitute for KCl.

[c] Some fractions from the purification of *B. stearothermophilus* that displayed topoisomerase I activity required the addition of 150mM NaCl or KCl as well as magnesium to be active (Yamamoto *et al.*, 1985).

[d] Is less active at lower concentrations.

[e] *Alcaligenes eutrophus* has a broad pH optimum of between 7.5-9.5 (Andera and Mikulik, 1990).

CHAPTER FIVE

PURIFICATION AND CHARACTERIZATION OF REVERSE GYRASE FROM *SULFOLOBUS SOLFATARICUS*

5.1 Introduction

The comments in the introduction of chapter four are also applicable to this chapter. *S. solfataricus* was chosen for reverse gyrase characterization as it could be grown in large quantities, showed little nuclease activity in crude protein preparations, and was different from *S. acidocaldarius*, the organism in which reverse gyrase has been most thoroughly examined. Furthermore *S. solfataricus* is phylogenetically distant from AN1 in terms of organisms that contain reverse gyrase and it was hoped that by using the same techniques in characterising *S. solfataricus* reverse gyrase as was used in characterising AN1 reverse gyrase, that any differences between the two enzymes would be revealed.

This chapter is divided into six sections (excluding the introduction) the first three of which contain results and discussion on the growth and purification of reverse gyrase using two different purification procedures. The fourth section examines some purification steps that were attempted but were not used for various reasons. The fifth section gives results and discussion on the characteristics of *S. solfataricus* reverse gyrase while the last section gives a general discussion of results from this chapter and a comparison with other reverse gyrase enzymes characterized.

5.2 Growth of *S. solfataricus*

Initial trials involving the growth of *S. solfataricus* cells in a 10l New Brunswick fermenter were disappointing with an average yield of 0.55gm/l of cells. Although aeration of the vessel appeared to be adequate this was one obvious factor which could effect the optimum yield of the organism. A second factor was the inclusion of stainless steel componentry in the fermenter

which is thought to inhibit growth of at least some thermophilic archaebacteria. Other factors which contributed to abandoning the use of this fermenter included the time required for autoclaving and the consistent attention needed to ensure that water levels in the water bath and media levels in the fermenter did not become low.

Most of the cells used in this study were grown in 500ml batches in 2l flasks in an orbital incubator. This technique normally yielded 1.1gm/l of cells at late log phase 16hr after inoculation, when a 5% inoculum was used. A typical growth curve for *S. solfataricus* is shown in figure 5.2.1 and compares cell growth as determined by optical density and cells/ml (determined using a cell counting chamber). The doubling time of cells in the log phase was 3.3hr (calculated between 4 to 15hr using OD_{600} results).

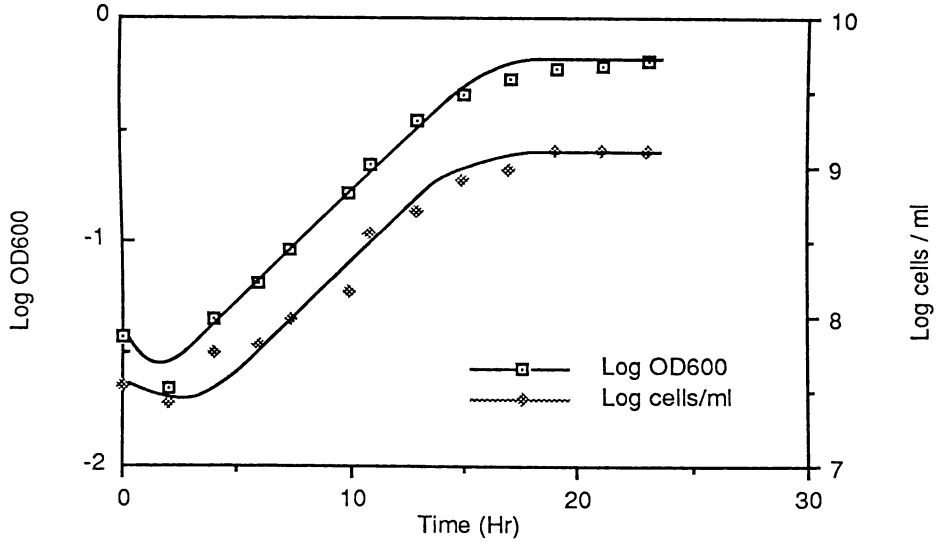


Figure 5.2.1 The growth curve of *S. solfataricus*. 500ml of *S. solfataricus* in 2l flasks was inoculated with a 5% inoculum of mid log cells and grown at 80°C with rotation at 120rpm in an orbital incubator. Cell growth was measured by optical density at 600nm and by direct cell counting using a Thoma-Hawksley counting chamber (see section 2.7).

5.3 Purification of reverse gyrase from *S. solfataricus* - Scheme I

Section 2.8 describes the procedures used in this purification. The yield of reverse gyrase from scheme I was 0.2% from 38gm of cells with a 50 fold purification. The details of this scheme are outlined in table 5.3.1 and elution profiles are shown in figures 5.3.1, 5.3.2, 5.3.3, 5.3.4, and 5.3.5. Fractions from purification steps were assayed for activity using the relaxation of negatively supercoiled pBR322 under standard reverse gyrase assay conditions (see section

2.3.1) as an indicator of activity.

Table 5.3.1 Purification of *S. solfataricus* reverse gyrase - Scheme I.

Purification step	Total protein (mg)	Total activity ($x10^4$ units[a])	Specific activity ($x10^3$ units/mg)	Yield (%)
Cell-free extract	3600	830	2.3	
Dialysed $(NH_4)_2SO_4$ pellet	1900	2760	14.3	100
CM Sepharose	307	1240	40	45
Heparin Agarose	68.2	400	59	15
MonoQ FPLC	10.1	68	67	2.5
MonoS FPLC	2.47	17	70	0.6
Phenyl-Superose FPLC	0.44	5	110	0.2

[a] One unit of activity was defined as the amount of protein required for 80% relaxation of negatively supercoiled DNA under standard assay conditions.

The steps in scheme I follow that used for purification of AN1 reverse gyrase except that the initial gel permeation step was replaced with cation exchange chromatography using CM Sepharose and the Blue Dextran Agarose step was replaced with Heparin Agarose chromatography. The successful steps using FPLC columns were left unchanged.

Cells were lysed under low ionic strength conditions using sonication. The cell free extract displayed a very low specific activity for reverse gyrase and a resultant total activity which was about three times less than that determined after the $(NH_4)_2SO_4$ precipitation step. For this reason reverse gyrase yield was determined from the $(NH_4)_2SO_4$ step. It was presumed that some inhibitory factor of reverse gyrase or the assay was in the crude cell extract but was not present after $(NH_4)_2SO_4$ precipitation. A likely candidate is genomic DNA which could competitively inhibit the reverse gyrase assay and which was removed during polymin P precipitation of DNA. Nuclease activity was not detected in crude cell fractions under the assay conditions used to determine reverse gyrase activity.

CM Sepharose chromatography reduced the amount of protein 6 fold with 26% of the loaded protein binding to the column. About one half of all activity was lost at this step with some coming off during column washing. Reverse gyrase eluted over a broad range of salt concentrations from 0.5-0.9M NaCl. The peak of activity (fraction 77) did not correspond to any of the peaks observed on the elution profile (figure 5.3.1).

During Heparin Agarose chromatography 34% of the protein bound to the column. A minority of reverse gyrase activity eluted from the column during washing while the majority of

activity was eluted during development of the linear salt gradient at a salt concentration of 0.4 to 0.8M NaCl. It was noticed that a contaminating DNA binding activity did not bind to the column and washed off under the starting conditions employed. Reverse gyrase activity peaked at fraction 13 (figure 5.3.2).

MonoQ FPLC bound 10% of the total protein loaded onto the column and reverse gyrase eluted off at the beginning of the gradient from 75 to 200mM NaCl. Reverse gyrase activity for this step peaked at fraction 7 (figure 5.3.3).

MonoS FPLC was not expected to purify reverse gyrase to a large extent as a cation exchange step with CM Sepharose had already occurred at the beginning of this scheme. Nevertheless a certain degree of purification did occur although the specific activity of reverse gyrase was not improved. 40% of protein loaded onto the MonoS column bound, and the majority of reverse gyrase activity eluted off at 250 to 300mM NaCl during development of the linear salt gradient. Reverse gyrase activity peaked within this range at fraction 6 (figure 5.3.4).

Phenyl-Superose FPLC bound 46% of the applied protein with reverse gyrase remaining firmly bound at this step with no activity eluting from the column wash. Reverse gyrase activity peaked at fraction 13 which corresponded to a single peak on the elution profile although a shoulder on the left hand side of this peak explains the presence of at least two protein species for the three fractions pooled (fractions 12-14) (figure 5.3.5). A sample of this pooled material was analysed on SDS-PAGE to determine its purity. The result (figure 5.3.6) showed the presence of five major bands all of low molecular weight ranging from 24.8 to 38.9kDa. One of these bands (33.7kDa) was a doublet and this was clearly evident in the silver stained version of the gel. This figure clearly shows the absence of any major band around 120 to 130kDa the accepted molecular weight for reverse gyrase. This material demonstrated cccDNA relaxing activity, however it was unfortunate that two dimensional gel electrophoresis was not performed at this stage to determine whether the extract could introduce positive supercoiling into cccDNA. An indication that positive supercoiling was catalysed by this fraction is seen by the presence of lighter secondary pBR322 topoisomers on 1D gels. These topoisomers are slightly separated from the predominant topoisomers (figure 5.3.7) and, in my experience, are only observed when positive supercoiling activity is present.

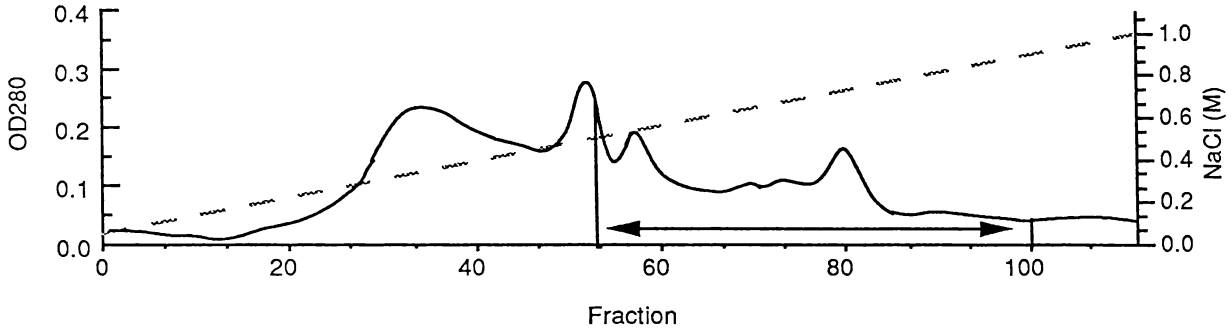


Figure 5.3.1 CM Sepharose chromatography of *S. solfataricus* reverse gyrase (Scheme I). Experimental details are described in section 2.8.2. Reverse gyrase activity was determined using the relaxation of cccDNA on agarose gels. The most active fractions, shown enclosed by vertical lines, were pooled and used for the next Heparin Agarose step.

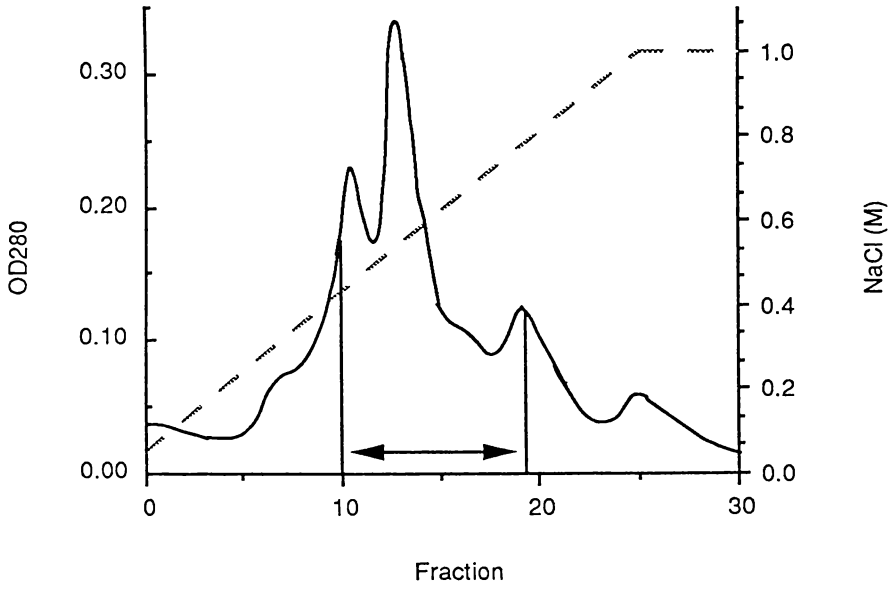


Figure 5.3.2 Heparin Agarose chromatography of *S. solfataricus* reverse gyrase (Scheme I). Experimental details are described in section 2.8.3. Reverse gyrase activity was determined as usual and the most active fractions, shown bounded by vertical lines, were pooled and used for the anion exchange FPLC step.

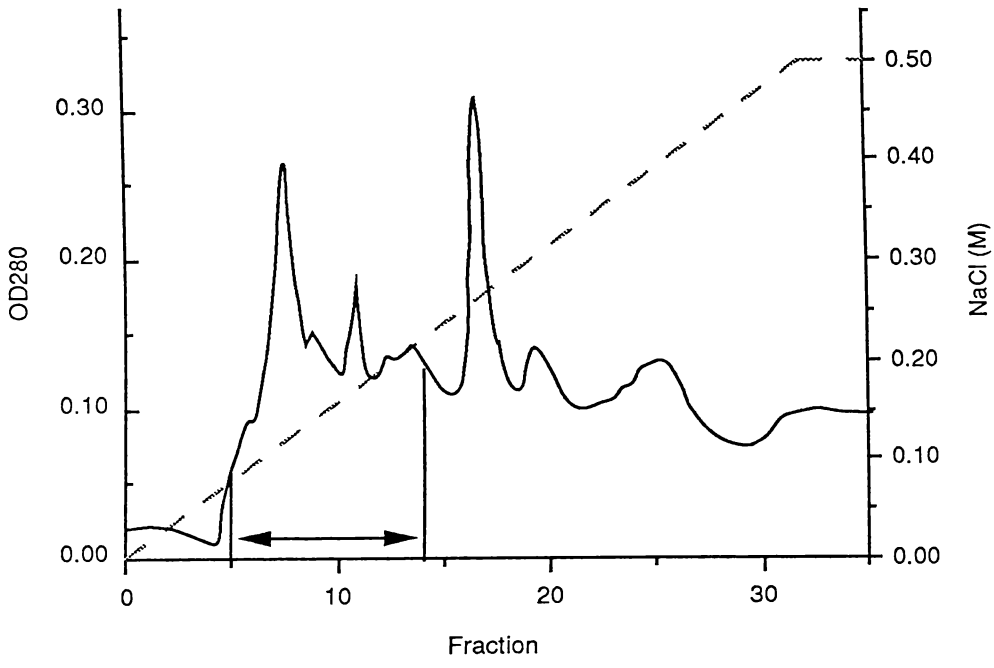


Figure 5.3.3 Anion exchange (MonoQ) FPLC of *S. solfataricus* reverse gyrase (Scheme I). Experimental details are described in section 2.8.4. Reverse gyrase activity was determined in the standard manner. The fractions contained within the vertical lines displayed the majority of activity and were used in cation exchange FPLC.

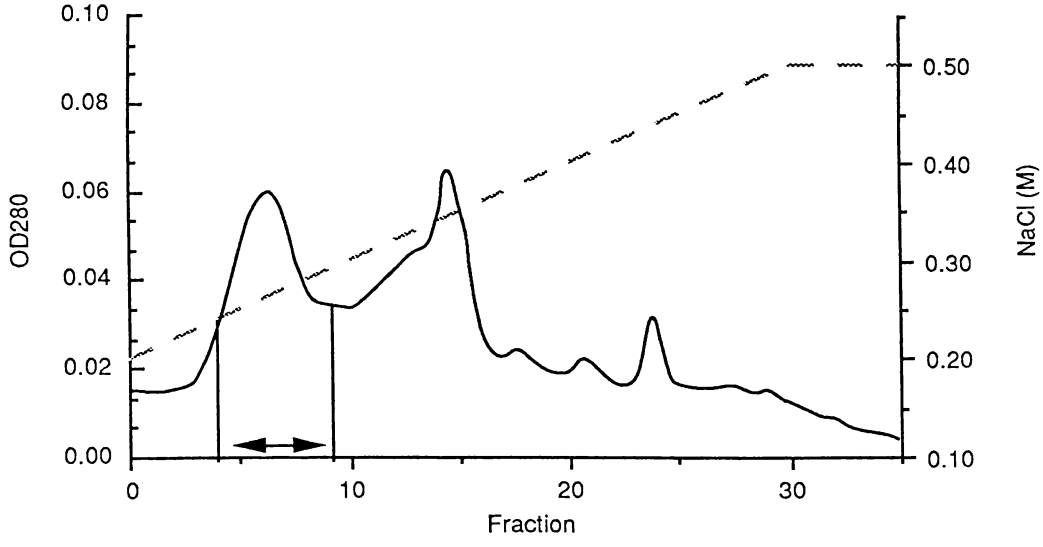


Figure 5.3.4 Cation exchange (MonoS) FPLC of *S. solfataricus* reverse gyrase (Scheme I). Experimental details are described in section 2.8.5. Active fractions (bounded by vertical lines) corresponded to a single peak on the elution profile and these were pooled and used for Phenyl-Superose FPLC.

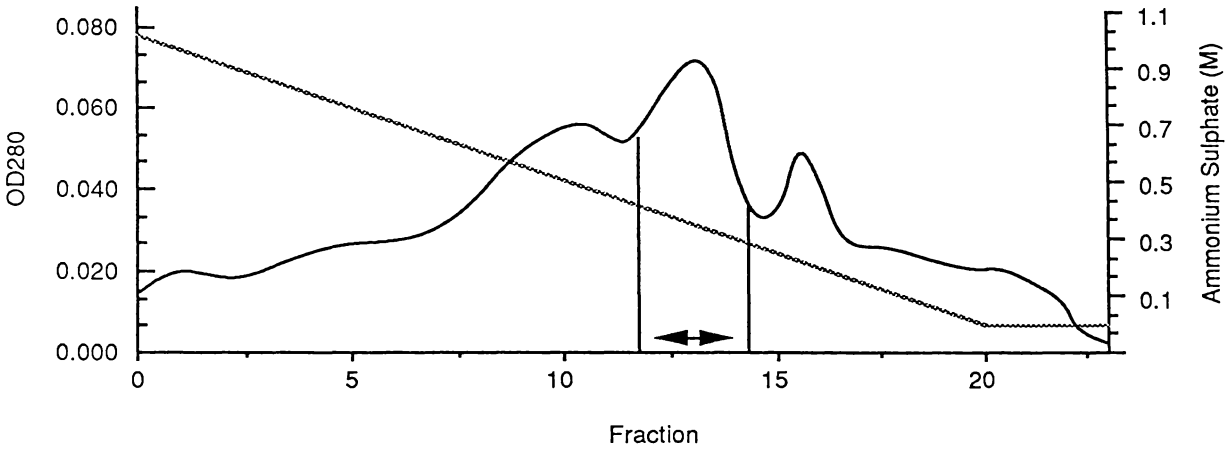


Figure 5.3.5 Hydrophobic interaction (Phenyl-Superose) FPLC of *S. solfataricus* reverse gyrase (Scheme I). Experimental details are described in section 2.8.6. The peak of reverse gyrase activity coincided with the major protein peak and the fraction (enclosed by vertical lines) was kept and its purity determined on SDS-PAGE.

In conclusion the final stage of purification with this scheme yielded a protein mixture which contained four major polypeptides all of low molecular weight and with no observable bands at 120 to 130kDa. It is inferred from this that either reverse gyrase of ~120kDa is present but in such small quantities that it cannot be detected on a silver stained polyacrylamide gel (though activity can be detected), or alternatively the reverse gyrase enzyme has undergone proteolytic degradation to a variety of lower molecular weight polypeptides, some or all of which are still able to relax negatively supercoiled DNA and probably catalyse the production of positively supercoiled DNA. A third trivial explanation not borne out by other researchers (Nadal *et al.*, 1988, Nakasu and Kikuchi, 1985) is that reverse gyrase is not monomeric but composed of subunits.

This material was not deemed suitable for further characterization of the enzyme so a second purification scheme was developed copying the initial steps of Nadal *et al.*, 1988 to remove the possibility of proteolytic cleavage during purification.

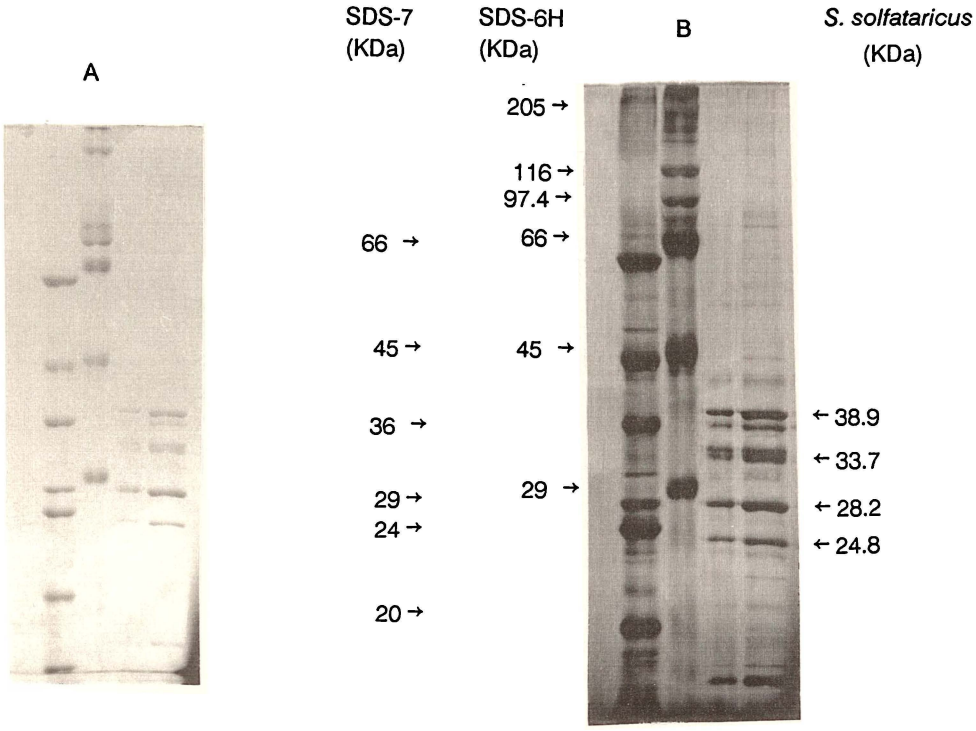


Figure 5.3.6 SDS-PAGE of purified *S. solfataricus* reverse gyrase (Scheme I). The resolving gel was 10% polyacrylamide and the stacking gel was 4% Samples were prepared by heating at 100°C for 5min in Laemmli sample buffer. Electrophoresis was at 15°C and carried out at 13mA in the stacking gel and 18mA in the resolving gel until the marker dye had reached 1cm from the bottom of the gel. The gel was visualized by Coomassie Blue staining and then further developed using silver staining.
Samples are: A, Coomassie Blue stained gel; B, silver stained gel. Lane 1 SDS-7 molecular weight markers (Sigma), lane 2 SDS-6H molecular weight markers (Sigma), lanes 3 and 4 purified sample from Phenyl-Superose FPLC.

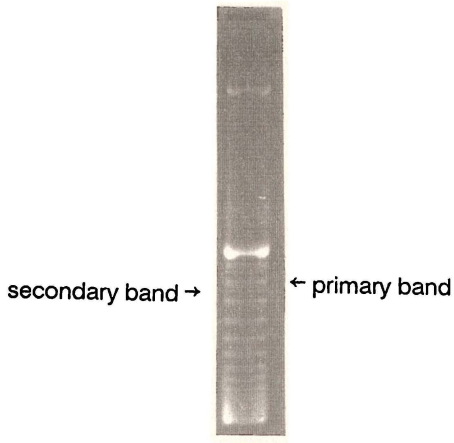


Figure 5.3.7 Purified reverse gyrase activity of Phenyl-Superose FPLC (Scheme I). Reverse gyrase was incubated under standard assay conditions and the resulting topoisomers of pBR322 run on a 1% agarose gel. Note the presence of lighter secondary bands on the topoisomer ladder which are indicative, in my experience, of positive supercoiling activity.

5.4 Purification of reverse gyrase from *S. solfataricus* - Scheme II

Section 2.9 describes the procedures used in this purification. The yield of reverse gyrase from scheme II was 0.9% from 44gm of cells. The details of this scheme are outlined in table 5.4.1 and elution profiles are shown in figures 5.4.1, 5.4.2, 5.4.3, and 5.4.4. Fractions from purification steps were determined for activity using the relaxation of negatively supercoiled pBR322 under standard assay conditions (see section 2.4.1) as an indicator of activity.

This scheme differs from AN1 reverse gyrase purification and *S. solfataricus* scheme I in that lysis and subsequent steps up to Phenyl Sepharose chromatography were carried out under high salt concentrations which are thought to inhibit proteolytic activity (Nadal *et al.*, 1988). Dye-binding chromatography was introduced as these columns had become available at this stage and during trials one in particular (Cibacron Navy F2R) had shown promise in the purification of reverse gyrase from the cell free lysate. The successful Phenyl-Superose FPLC step was retained from previous schemes despite the fact that it acts in a similar fashion to Phenyl Sepharose chromatography, and a final step using gel permeation FPLC was introduced to separate the remaining protein species by molecular weight. One important aspect of this purification scheme is that all steps, with the exception of dye-binding chromatography, can proceed at high ionic strength. This minimises the possibility of proteolytic degradation and reduces the time required for desalting and buffer exchange between chromatographic procedures.

Table 5.4.1 Purification of *S. solfataricus* reverse gyrase - Scheme II.

Purification step	Total protein (mg)	Total activity ($\times 10^4$ units[a])	Specific activity ($\times 10^3$ units/mg)	Yield (%)
Cell free extract	5300	1300	2.5	100
Dialysed $(NH_4)_2SO_4$ pellet	3500	970	2.8	75
Phenyl Sepharose	272	360	13	28
Cibacron Navy F2R	17	36.5	21	2.8
Phenyl-Superose FPLC	1.65	32	190	2.5
Gel permeation FPLC	0.2	12	600	0.9

[a] One unit of enzyme was defined as the amount of protein required for 80% relaxation of negatively supercoiled DNA under the standard assay conditions.

During this purification the loss of reverse gyrase activity was monitored in the initial steps. It was found that polymin P precipitation resulted in an 8% loss of activity from the original material while the precipitate from ultracentrifugation accounted for a further 5% loss. Desalted material from the supernatant of the 70% $(NH_4)_2SO_4$ cut displayed no reverse gyrase activity

while a major portion of all lost activity in the first stages was found in the 35% $(NH_4)_2SO_4$ precipitate (13%).

Phenyl Sepharose chromatography was a successful step in terms of removing the bulk of unwanted protein and increasing the specific activity of reverse gyrase (5x). 55% of the protein loaded onto the column did not bind while 23% was eluted from the column during washing with 0.25M NaCl and a further 10% of protein was removed with 30% ethylene glycol, the starting buffer for gradient development. Reverse gyrase was eluted over a broad range of fractions from the column during development of the linear 30-60% ethylene glycol gradient (figure 5.41). It was thought that the poor resolution of reverse gyrase during gradient development was primarily due to overloading of the column as 35mg of protein per ml of Phenyl Sepharose was loaded per run. The capacity of Phenyl Sepharose is about 15-20mg of protein per ml of gel as determined by the binding of human serum albumin with a molecular weight of 68kDa (1989 Sigma Catalogue, Sigma Chemical Company). Overloading of this column undoubtedly contributed to the poor yield of reverse gyrase activity achieved by this step (60% loss of activity) with the majority of lost activity not binding to the column in the first instance, while little activity was eluted from the column during the washing stages of this procedure. This compares with a loss of 10-30% of activity experienced by Nadal *et al.*, 1988 using an identical protocol but with a protein loading of 10mg protein per ml of gel. Some proteins remained bound to the column after gradient development to 60% ethylene glycol and these were removed by Triton X-100.

Dye-binding chromatography with Cibacron Navy F2R proved to a be a disappointing step with little increase in specific activity for reverse gyrase and a large drop in the yield of the enzyme. The reason for the poor performance of this step is not known and it appears that the cell free lysate used in original trials with the column did not give equivalent results with the partially purified material used in this step. 14% of protein loaded onto the column bound and the majority of reverse gyrase activity was eluted during development of the linear gradient at a salt concentration of 420-800mM NaCl, peaking at fractions 25 to 28 (fraction 5.4.2).

During Phenyl-Superose FPLC, reverse gyrase remained bound to the column with no activity eluting off during washing. Reverse gyrase activity eluted from the column between 450 to 250mM $(NH_4)_2SO_4$ with activity peaking at fraction 20 (figure 5.4.3).

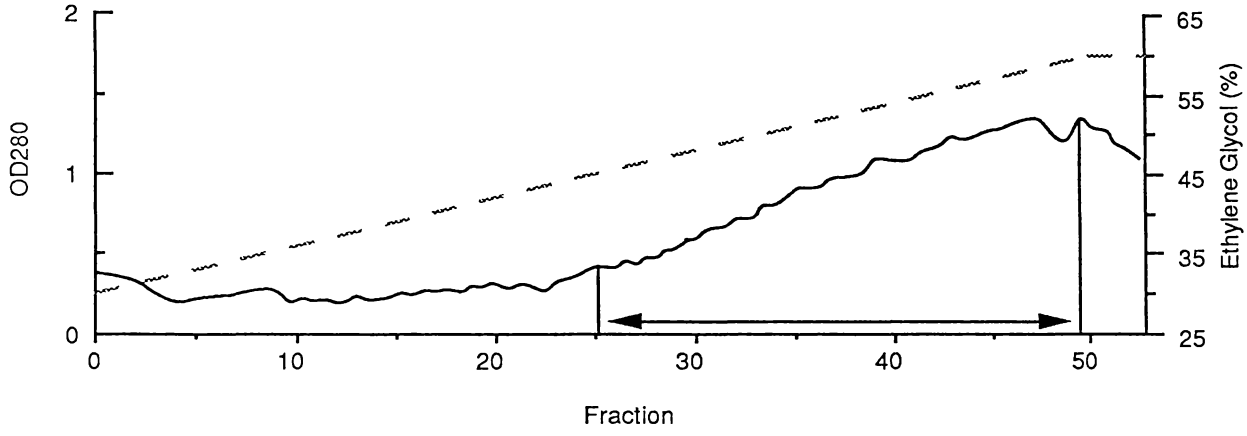


Figure 5.4.1 Phenyl Sepharose chromatography of *S. solfataricus* reverse gyrase (Scheme II). Experimental details are described in section 2.9.2. Reverse gyrase activity was determined using the relaxation of cccDNA on agarose gels. Active fractions are shown bounded by vertical lines and these were taken into the dye-binding step.

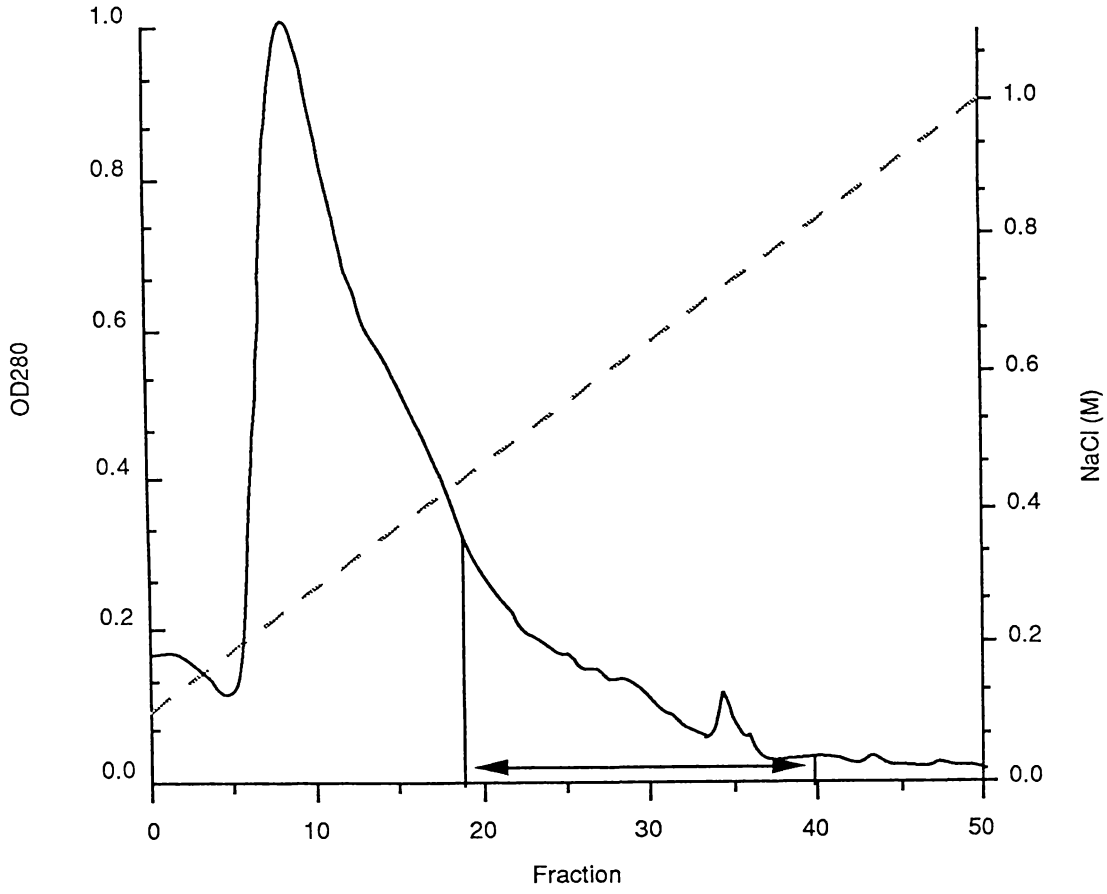


Figure 5.4.2 Dye-binding chromatography (Cibacron Navy F2R) of *S. solfataricus* reverse gyrase (Scheme II). Experimental details are described in section 2.9.3. Reverse gyrase activity was assayed as usual and the most active fractions, bounded by the vertical lines, pooled and used for the next Phenyl-Superose FPLC step.

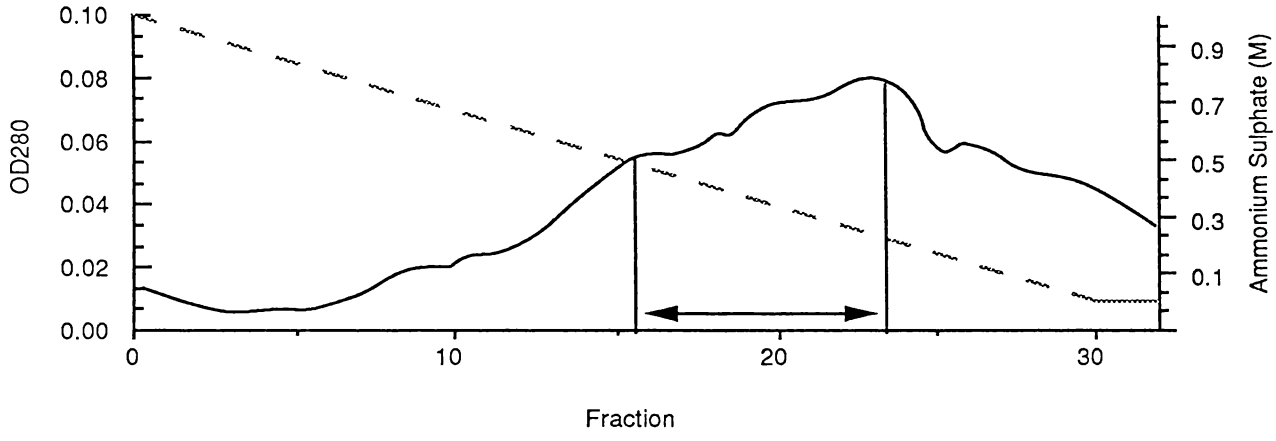


Figure 5.4.3 Hydrophobic interaction (Phenyl-Superose) FPLC of *S. solfataricus* reverse gyrase (Scheme II). Experimental details are described in section 2.9.4. Reverse gyrase activity was assayed as usual and the peak of activity was determined to lie between the vertical lines. These fractions were used for the next step involving gel permeation.

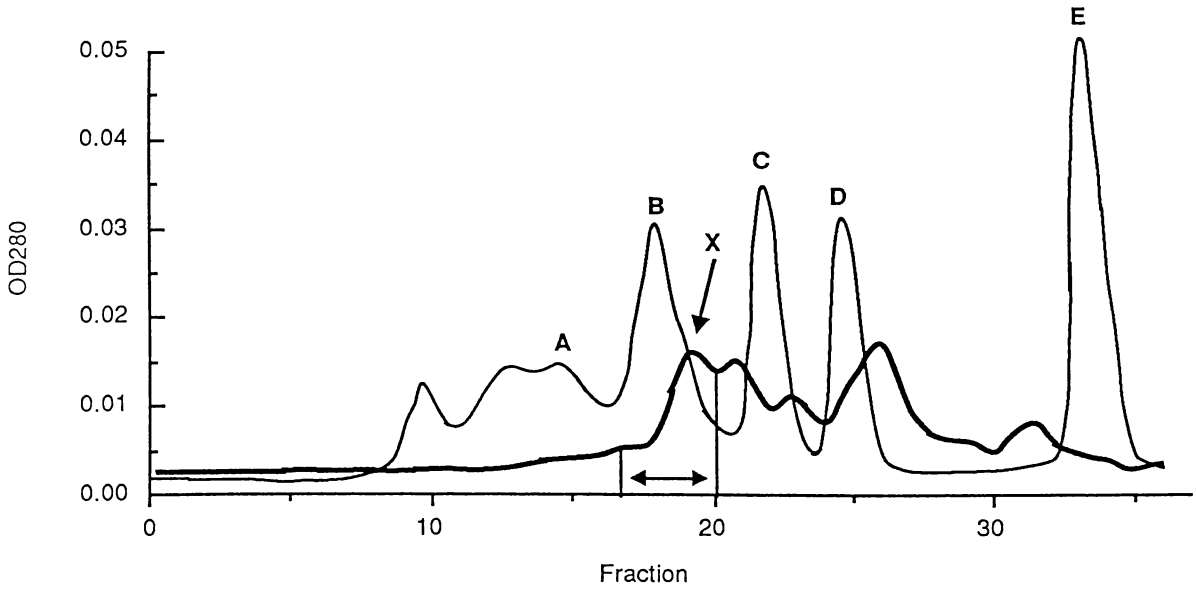


Figure 5.4.4 Gel permeation FPLC of *S. solfataricus* reverse gyrase (Scheme II). Experimental details are described in section 2.9.5. At this stage reverse gyrase activity corresponded to a single peak on the elution profile. The fractions corresponding to this peak were pooled, desalted, and freeze dried to yield a powder containing highly active reverse gyrase.

The material pooled from Phenyl-Superose FPLC was used in consecutive runs on gel permeation FPLC. All runs gave an identical elution profile and reverse gyrase activity eluted at the same point in all runs. At this stage activity corresponded with a single peak on the elution profile which gave a molecular weight for the enzyme of 120,200Da when compared with molecular weight standards run under the same conditions (figure 5.4.4). SDS-PAGE was run on fractions from this column and showed (figure 5.4.5) a high molecular weight polypeptide of 122,500Da corresponding to the peak of activity observed during gel permeation FPLC (lane 5, fractions 17-19). Material from this peak was freeze dried and contained reverse gyrase activity as determined using two dimensional gel electrophoresis figure 5.4.6.

The purity of this sample was analysed using SDS-PAGE. The result (figure 5.4.7) shows a major band of high molecular weight (122.5kDa) which comprises about 12% of the total protein in this track as determined by laser scanning densitometry. The molecular weight determined using gel permeation FPLC corresponds to this major band which was taken to be the reverse gyrase polypeptide. Known quantities of protein were loaded onto the gel shown in figure 5.4.7 (assuming 1ul per track was loaded using the PhastGel system) and from this it was determined that a total of 24ug of the 200ug of protein in the last step corresponded to reverse gyrase. This gives reverse gyrase a specific activity of $6x10^6$ units/mg in a totally pure form which is in close agreement to the specific activity found by Nadal *et al.*, 1988 for their totally pure reverse gyrase ($5.5x10^6$ units/mg when a conversion factor of 0.625 is applied to convert 50% relaxation of cccDNA for 1 unit to 80% relaxation of cccDNA for 1 unit).

It was considered that the freeze dried material was of sufficient purity to initiate characterization of the enzyme. A sample of this material was sent to the University of Auckland (Department of Biochemistry) for N terminal amino acid sequencing of the major polypeptide. The methodology used in sequencing involved running the sample on SDS-PAGE, transferring the protein onto a nylon membrane and cutting and eluting the polypeptide band of interest. Coomassie Blue staining of the protein apparently does not affect amino acid analysis. Finally N terminal sequencing of the polypeptide was attempted using Edman degradation methodology on an automated amino acid sequencer. Unfortunately it appeared that the N terminal sequence was blocked (D. Christie, University of Auckland, New Zealand, personal communication) and

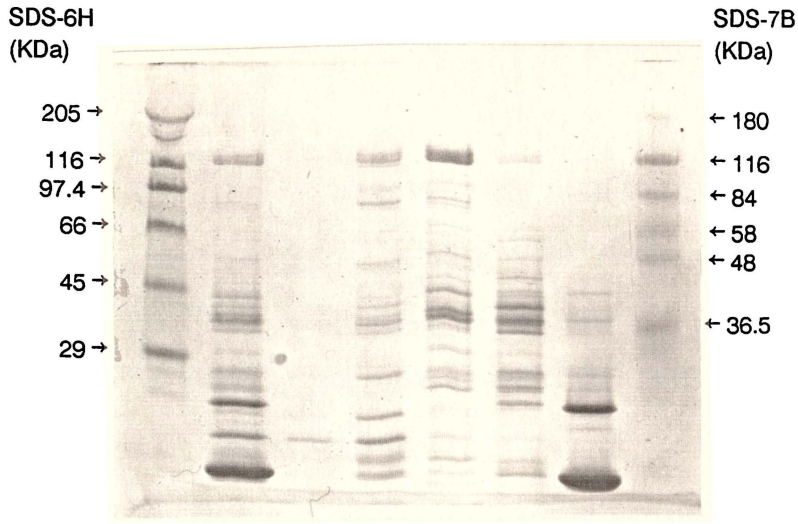


Figure 5.4.5 SDS-PAGE on fractions eluted off gel permeation FPLC (Scheme II). Fractions were analyzed on a PhastGel (10-15% polyacrylamide gradient) using the PhastSystem separation and control unit (Pharmacia) coupled to the PhastSystem development unit (Pharmacia). The gel was run and stained with Coomassie Blue using procedures developed for this system (PhastSystem operations manual, Pharmacia). Samples are, from left to right; lane 1, SDS-6H molecular weight markers (Sigma); lane 2, sample applied onto gel permeation FPLC; lane 3, fractions 9-12; lane 4, fractions 13-16; lane 5, fractions 17-19; lane 6, fractions 20-23; lane 7, fractions 24-27; lane 8, SDS-7B molecular weight markers (Sigma).

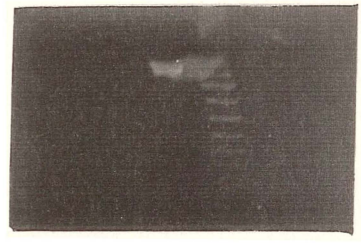


Figure 5.4.6 Two dimensional agarose gel electrophoresis on pBR322 treated with freeze dried material containing purified reverse gyrase from the final gel permeation FPLC step. This material at the concentrations used and under standard assay conditions produced a ladder of pBR322 topoisomers which were almost entirely positively supercoiled.

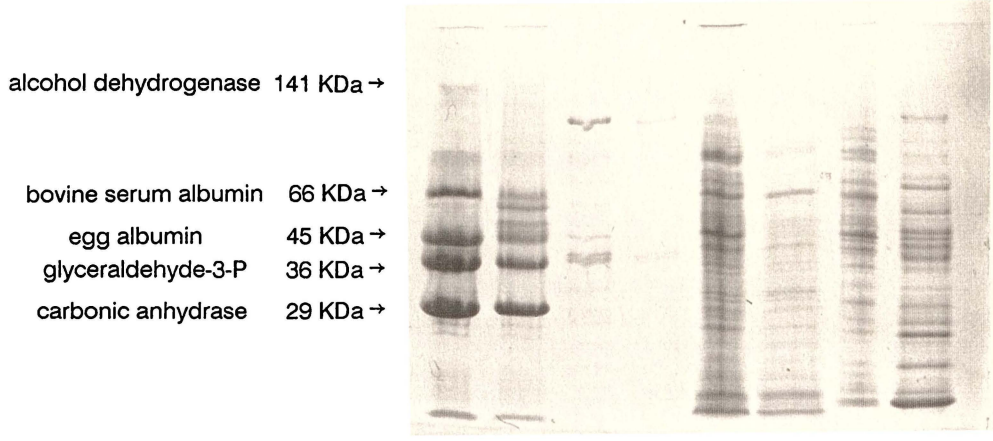


Figure 5.4.7 SDS-PAGE on fractions from steps used in the purification protocol of Scheme II. Fractions were analysed on a 10-15% polyacrylamide PhastGel using the procedures and system outlined in the text of figure 5.4.5. Samples are, from left to right; lanes 1 and 2, protein markers (a) alcohol dehydrogenase 0.2ug (lane 1) 0.05ug (lane 2), (b) bovine serum albumin 0.5ug (lane 1) 0.1ug (lane 2), (c) egg albumin 1.0ug (lane 1) 0.2ug (lane 2), (d) glyceraldehyde-3-P 1.5ug (lane 1) 0.4ug (lane 2), (e) carbonic anhydrase 2.0ug (lane 1) 0.8ug (lane 2); lane 3, freeze dried material off gel permeation FPLC (0.7mg freeze dried extract in 14ul of dilution buffer); lane 4, 1/5 dilution of freeze dried extract from lane 3; lane 5, *S. solfataricus* crude cell free lysate; lane 6, *S. solfataricus* protein after polymin P and $(NH_4)_2SO_4$ precipitation steps; lane 7, *S. solfataricus* protein after Phenyl Sepharose purification; lane 8, partially purified reverse gyrase after dye-binding chromatography. The amounts of protein standards loaded onto the PhastGel were calculated by assuming a loading volume per lane of 1ul.

further work was not attempted to elucidate the amino acid sequence of the enzyme. An obvious extension of this work would be to obtain an internal peptide sequence of reverse gyrase by proteolytic digestion of the polypeptide followed by SDS-PAGE and sequencing of a fragment. Such a sequence could be used to prepare a nucleic acid probe which could then be used to screen a gene library of *S. solfataricus* for the reverse gyrase gene.

5.5 Miscellaneous trials for purifying reverse gyrase

Apart from the purification steps outlined in schemes I and II, other chromatographic and electrophoretic procedures were tested to determine their efficacy in purification of reverse gyrase. Some of these procedures are briefly discussed below:

(a) Gel permeation HPLC. A TSK-gel G3000SWG HPLC column (60x2.15cm) (Toyo Soda Manufacturing Co. Ltd) was trialed for reverse gyrase purification. At low ionic strengths the enzyme apparently bound to the column so that activity was eluted over a broad range of molecular weights. Under conditions of high salt (0.5M ammonium acetate) and 5% methanol, which reduce ionic and hydrophobic interactions respectively, reverse gyrase was eluted as a single tight peak. The activity recovered using this procedure was poor (~40%).

(b) Gel permeation under denaturing conditions. The rationale behind this trial was that the monomeric molecular weight of reverse gyrase was of a large enough size to facilitate good separation from the bulk of the protein using gel permeation provided that multimeric complexes were eliminated. Trials using Sepharose CL-6B gel permeation chromatography under conditions of 6M Guanidine-HCL with the reverse gyrase sample in the same denaturant resulted in poor recovery of activity despite the fact that initial attempts at renaturing reverse gyrase from a denatured state showed some success. Furthermore wheat germ type I topoisomerase can be successfully renatured after denaturation in 6M Guanidine-HCL (Hager and Burgess, 1980).

(c) Reverse phase chromatography. Reverse phase chromatography using a Brownlee RP-18 silica based HPLC column and developed with a 5-50% linear methanol gradient, was used in an attempt to purify reverse gyrase. The advantages of reverse phase chromatography include extremely good resolution of protein species and this was observed during this trial as determined by an elution profile which contained numerous (>40) peaks. The main

disadvantage of this system was the requirement for large proportions of organic solvent (usually methanol or acetonitrile) in the buffer in order to elute protein species. Initial trials of reverse gyrase stability in organic solvents showed that it remained active when incubated at room temperature in methanol concentrations of 35% or below for 5hr or acetonitrile concentrations of 30% or below for 5hr. Reverse gyrase was not recovered in an active form from reverse phase chromatography using either methanol or acetonitrile as the eluting agent and it was concluded that reverse gyrase elutes from the column at a relatively late stage in the gradient.

(d) Preparative isoelectric focusing. The protocol for this method is described in section 2.11.2 and gave good results with isoelectric focusing markers (figure 5.5.1). However this method does not achieve a good recovery of reverse gyrase activity (<20% in the several instances in which it was attempted). This technique was used in an attempt to resolve the bands seen on SDS-PAGE after the final Phenyl-Superose FPLC step in scheme I. The result was a doublet band with a pI of 5.2 on preparative IEF (figure 5.5.2) which displayed reverse gyrase activity. When this band was rerun on SDS-PAGE little purification from the original sample was observed, 3-4 major bands still being present on the silver stained gel.

Preparative isoelectric focusing on a smaller scale could also be achieved on the PhastGel apparatus (Pharmacia) using PhastGels (Isoelectric focusing pH3-9) from which activity could be isolated corresponding to a single band (see figure 5.6.15).

(e) Renaturation of enzyme activity after electrophoresis involving SDS-PAGE. This method was attempted by following the protocol described by Hager and Burgess, 1980 who successfully renatured wheat germ DNA topoisomerase after running it in a denatured form on SDS-PAGE. The advantage of such a method is that the exact polypeptide which represents activity can be determined from SDS-PAGE even in semi-purified material. However for this procedure to succeed the enzyme in question must be monomeric and be amenable to renaturation after denaturation by SDS. In three separate trials using this procedure, reverse gyrase, although being a monomeric enzyme (Nadal *et al.*, 1988), could not be renatured into an active form.

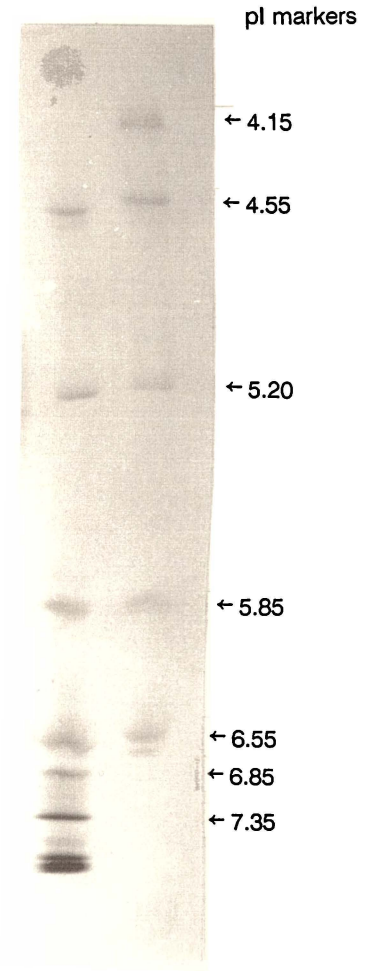


Figure 5.5.1

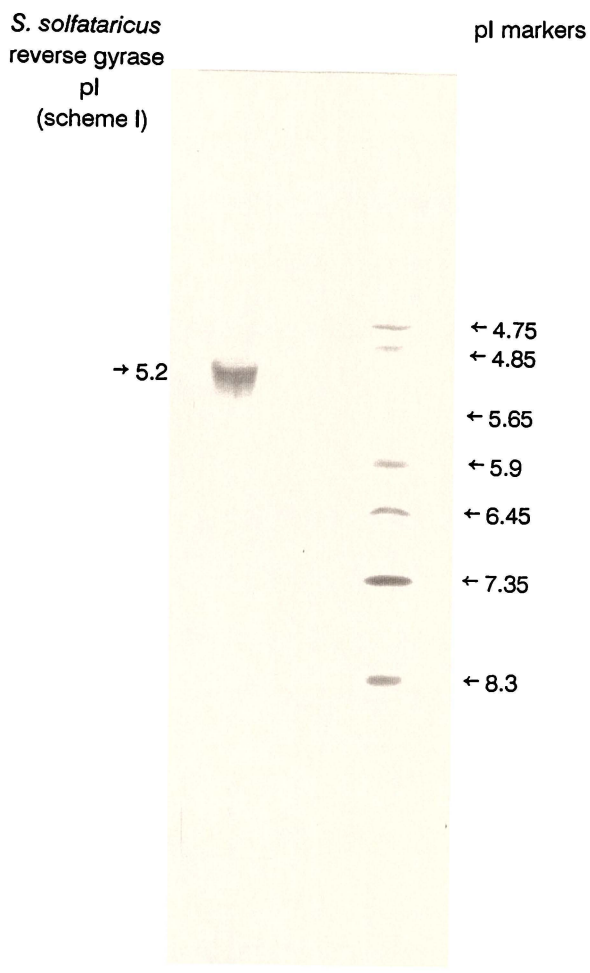


Figure 5.5.2

Figure 5.5.1 Isoelectric focusing (pH3-10) of Pharmacia standards using an agarose gel matrix. Experimental details are described in section 2.11.2. The left hand lane contains gold standards with markers from pI3-10 (Pharmacia) while the right hand lane contains red standards with markers from pI2.5-6.5 (Pharmacia).

Figure 5.5.2 Isoelectric focusing (pH3-10) of purified reverse gyrase from Scheme I after Phenyl-Superose FPLC, and pI markers. Experimental details are described in section 2.11.2. Lanes, from left to right, contain: lane 1, purified sample with reverse gyrase activity (Scheme I); lane 2, isoelectric point markers pI3-10 (gold standards, Pharmacia); lane 3, isoelectric point markers pI4.7-10.6 (BDH).

5.6 Characterization of reverse gyrase from *S. solfataricus*

Characterization of reverse gyrase from *S. solfataricus* was carried out using the purified freeze dried enzyme (~120kDa) from scheme II and this extract after reconstitution produced positive supercoiling of pBR322. However for the purpose of characterization of the enzyme, the reconstituted extract was diluted with 50ug/ml bovine serum albumin, 50mM Tris/HCl (ph7.5), and 1mM spermidine to a point where the relaxation of the negatively supercoiled substrate could be monitored on one dimensional gels using scanning laser densitometry. This allowed enzyme activity to be readily quantified by determining the extent of the reduction of form I pBR322 to other topoisomers.

The activity of reverse gyrase from *S. solfataricus* was determined under different assay conditions by varying the parameter in question and keeping all other factors constant. The standard reverse gyrase assay was performed at 80°C for 10min and contained 50mM MOPS/NaOH pH6.6 (20°C), 0.2M NaCl, 10mM $MgCl_2$, 1mM spermidine, 1mM DTT, 1mM ATP, and 0.4ug negatively supercoiled pBR322.

5.6.1 Temperature

Reverse gyrase was assayed under standard conditions with MOPS/NaOH as the buffer. MOPS was chosen as it exhibits a small pH change when subjected to varying temperature (pKa/°C of -0.006). Three MOPS/NaOH buffers were made to cover the temperature spectrum investigated; 50mM MOPS/NaOH pH6.35 (20°C) for assays between room temperature to 50°C, 50mM MOPS/NaOH pH6.5 (20°C) for assays between 55 to 80°C, and 50mM MOPS/NaOH pH6.7 (20°C) for assays between 85 to 110°C. The use of these three buffers enabled the pH variation due to temperature to be controlled to within 0.18 pH units for all assays. It was thought that such a small pH change was not likely to influence the results of reverse gyrase activity due to temperature. The 110°C assay was accomplished using an oil bath and tightly capped 400ul capacity ependorf tubes, which reduced evaporation of the sample. The results of this experiment are shown in figures 5.6.1 (graph) and 5.6.3 (gel) and clearly show a temperature optimum for this enzyme at 80°C under the assay conditions used. As explained in the previous chapter the temperature optimum probably reflects the two opposing factors of increasing

catalytic activity and increasing denaturation of the enzyme with temperature. Enzyme activity was noticed, under the assay conditions and enzyme concentration used, in a range from 70°C to 90°C although this upper limit is doubtful as a loss of negative supercoils was noticed at higher temperatures; however, no ladder of topoisomers was seen. Whether this was due to denaturation of pBR322 under high temperature or enzyme activity was not ascertained.

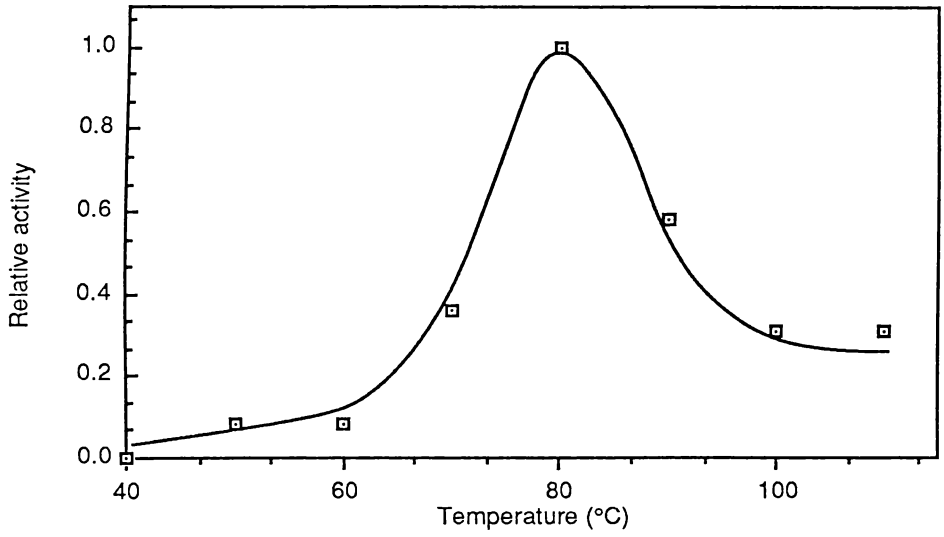


Figure 5.6.1 The effect of temperature on reverse gyrase activity. Standard reverse gyrase assay conditions were used throughout this experiment, a change in temperature being the only variable. Standard assay conditions were a 10min incubation at 80°C in 50mM MOPS/NaOH pH6.6 (20°C), 0.2M NaCl, 10mM $MgCl_2$, 1mM spermidine, 1mM DTT, 1mM ATP and 0.4ug negatively supercoiled pBR322.

5.6.2 pH

Standard assay conditions were used in this experiment with the buffer changed to achieve the required pH. Two buffer systems were used; MOPS/NaOH for final pH's of between 6.1 to 7.5, and MES/NaOH for final pH's of between 4.8 to 6.1. The buffers were adjusted at room temperature to compensate for dilution and temperature effects in order to achieve the desired pH at 50mM buffer concentrations and 80°C (see section 4.3.2). Optimum reverse gyrase activity was found at a pH of 6.3 to 6.4 under the assay conditions employed with activity being detected in a range from pH5.4 to 6.9. Figures 5.6.2 (graph) and 5.6.4 (gel) show the results of this experiment.

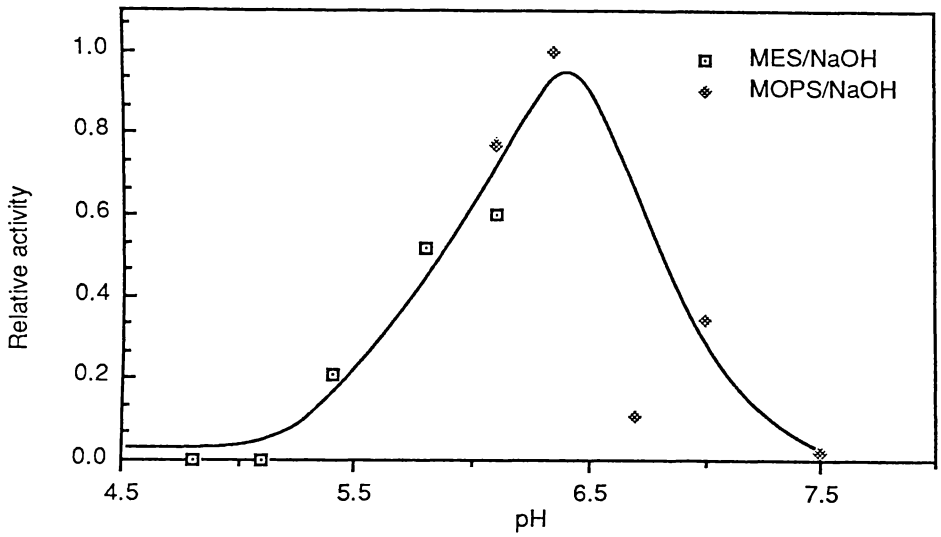


Figure 5.6.2 The pH optimum of *S. solfataricus* reverse gyrase. Standard assay conditions were maintained throughout this experiment while the pH was varied. A MES/NaOH buffer was used when assaying at a pH of 4.8-6.1 while a MOPS/NaOH buffer was used for assays between 6.1-7.5.

5.6.3 Salt and Magnesium

The effect of monovalent cations on activity was determined under standard assay conditions with NaCl or KCl concentrations ranging from 0 to 500mM. Salt was required for activity (figures 5.6.7 (graph) and 5.6.5 (gel)) with both NaCl or KCl being equally effective. Optimum activity in both cases was found at 200mM while activity could be discerned at salt concentrations between 100 to 350mM. No processive action at low salt concentrations was evident on one dimensional gels and the possession of this activity, found by Forterre *et al.*, 1985 in the reverse gyrase of *S. acidocaldarius*, was not further investigated.

Standard assay conditions were again used to determine the effect of magnesium on reverse gyrase activity. The results of this experiment can be seen in figures 5.6.8 (graph) and 5.6.6 (gel) and show that optimum reverse gyrase activity under these assay conditions is at 10mM $MgCl_2$ while activity was seen in a range from 1 to 32mM $MgCl_2$.

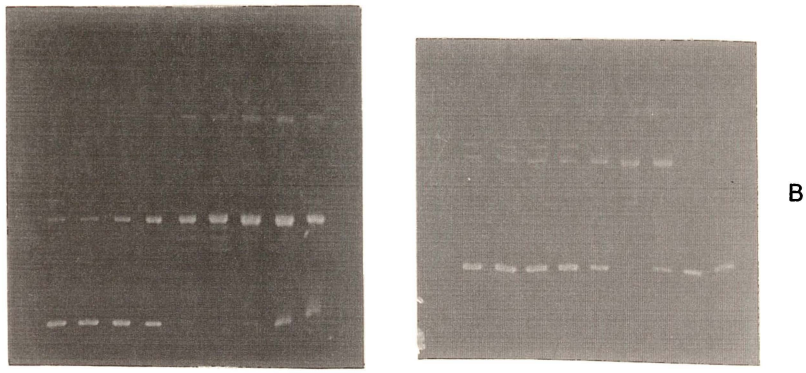


Figure 5.6.3 Agarose gel showing the effect of temperature on reverse gyrase activity. Gel A lanes, from left to right, were assayed at; room temperature, 40, 50, 60, 70, 80, 90, 100, and 110°C. Gel B lanes, from left to right, were assayed at; 55, 60, 65, 70, 75, 80, 85, 90 and 95°C.

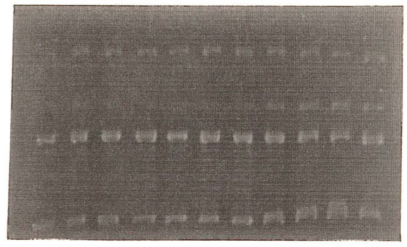


Figure 5.6.4 Agarose gel showing the pH optimum of *S. solfataricus* reverse gyrase. Lanes, from left to right, were assayed at a pH of; 7.5, 7, 6.7, 6.35, 6.1, 6.1, 5.8, 5.4, 5.1, and 4.8. The first five tracks (pH7.5-6.1) were assayed in a MOPS/NaOH buffer while the last five tracks (pH6.1-4.8) were assayed in a MES/NaOH buffer.

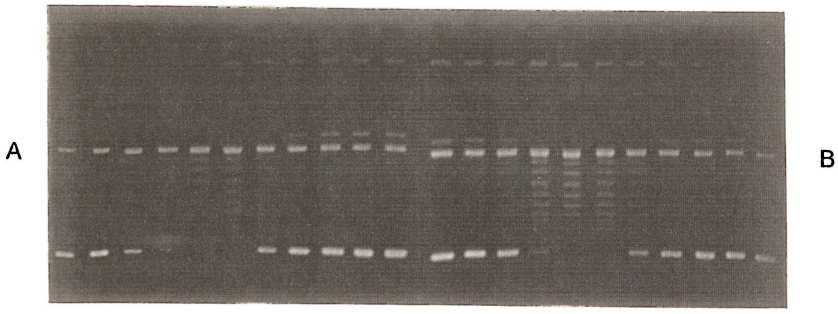


Figure 5.6.5 Agarose gel showing optimum salt concentrations for *S. solfataricus* reverse gyrase activity. Gel A shows the effect of NaCl on activity, gel B shows the effect of KCl on activity. For both gels the lanes, from left to right, were assayed at; 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500mM salt.

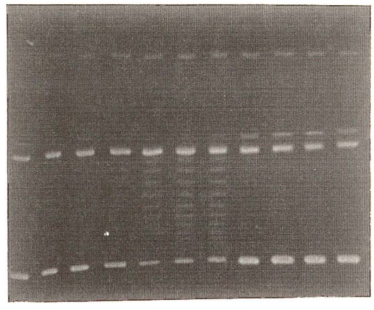


Figure 5.6.6 Agarose gels showing the effect of Mg^{2+} on the activity of *S. solfataricus* reverse gyrase. Lanes, from left to right, were assayed at; 0, 1, 2, 4, 8, 12, 16, 32, 64, 128, and 256mM $MgCl_2$.

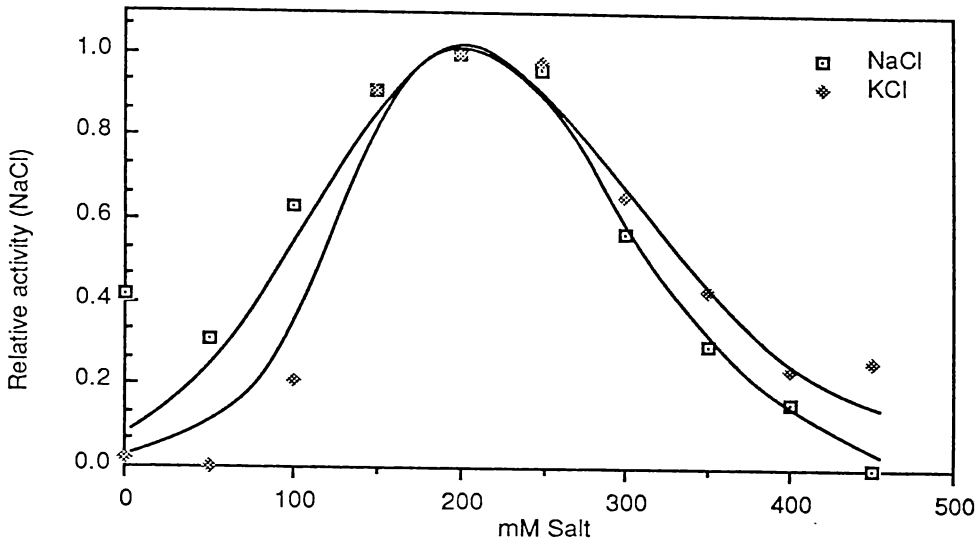


Figure 5.6.7 Optimum salt concentration for *S. solfataricus* reverse gyrase activity. Assays were performed under standard reverse gyrase conditions the only variation being salt concentration.

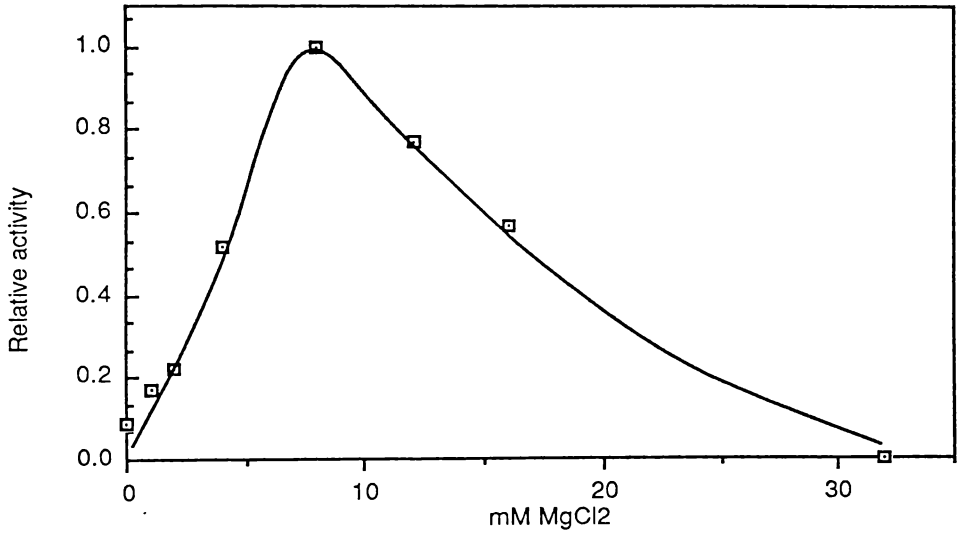


Figure 5.6.8 Effect of varying magnesium concentrations on *S. solfataricus* reverse gyrase activity. Reverse gyrase assays were carried out using standard assay conditions with a variation in $MgCl_2$ concentration.

5.6.4 ATP

The effect of ATP on reverse gyrase activity was determined using standard assay conditions and varying the concentration of ATP. The results, shown in figures 5.6.9 (graph) and 5.6.11 (gel), show that ATP is essential for activity, which will occur even at low ATP concentrations (1uM), although positive supercoiling of the DNA substrate is unlikely to occur at

these levels. This experiment showed an increase in activity over the whole range of ATP concentrations used up to 1.25mM ATP. This is in contrast to the result obtained for AN1 reverse gyrase (section 4.3.4) in which an increase of activity was not evident at ATP concentrations of 128uM or higher on 1D gels. The reason for the discrepancy between these two results is not known although one possibility is the presence of a contaminating ATPase activity in the *S. solfataricus* sample which was not totally pure. Such an activity could compete with the ATPase domain on reverse gyrase which would increase the concentration of ATP needed in the assay mix for full reverse gyrase activity to occur.

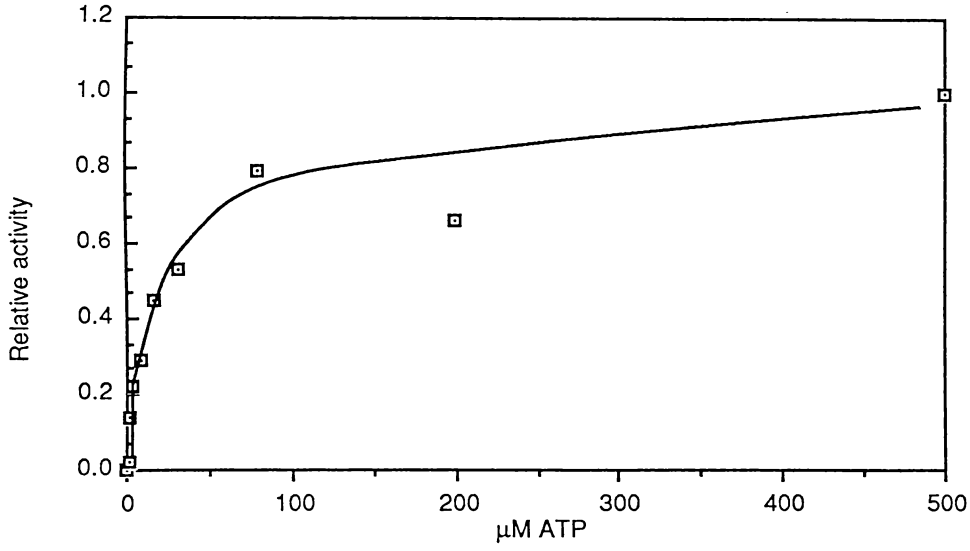


Figure 5.6.9 Effect of varying ATP concentrations on *S. solfataricus* reverse gyrase. Reverse gyrase assays were carried out using standard assay conditions with a variation in ATP concentrations.

5.6.5 Thermostability of *S. solfataricus* reverse gyrase at 75°C

The thermostability of reverse gyrase in dilution buffer (50ug/ml bovine serum albumin, 50mM Tris/HCl pH7.5, and 1mM spermidine) at 75°C was investigated in the presence and absence of 200ug/ml double-stranded calf thymus DNA. For this experiment the reverse gyrase solution was five times as concentrated as that used in previous experiments. In order to prevent evaporation over long incubation periods, sealed glass capillary tubes were trialled. However these proved unsuitable due to the difficulty in extracting the solution from the capillary after incubation and small sealed ependorf tubes with a 400ul capacity were used instead. These were

centrifuged every 40min to precipitate evaporated material from the reverse gyrase solution. The results of this experiment shown in figures 5.6.10 (graph) and 5.6.12 (gel), must be interpreted with caution for several reasons. First, as previously explained for the denaturation experiments involving AN1 reverse gyrase, a considerable component of denaturation undoubtedly occurs while the assay for activity is being run. This would especially be the case for this enzyme where denaturation of the enzyme occurs at 75°C and the standard assay conditions used to determine activity include a 10min incubation at 80°C. A second reason for interpreting this experiment with caution is the observed unreliability of this technique, for example the first two lanes in the gel which show denaturation in the presence of DNA appear to exhibit less activity than the next four lanes with longer incubation times.

It is concluded that these experiments can only give a rough indication of reverse gyrase thermostability. The half life of *S. solfataricus* reverse gyrase at 75°C (including an 80°C 10min incubation) in the absence of DNA was about 30min while in the presence of DNA it appeared that thermostability was increased to about 60min. The denaturation curve obtained in the presence of DNA was unusual, with a sudden drop in activity between 40 to 80min, so that the activity seen after this time was less than that observed in the absence of DNA. I can think of no explanation for this phenomenon.

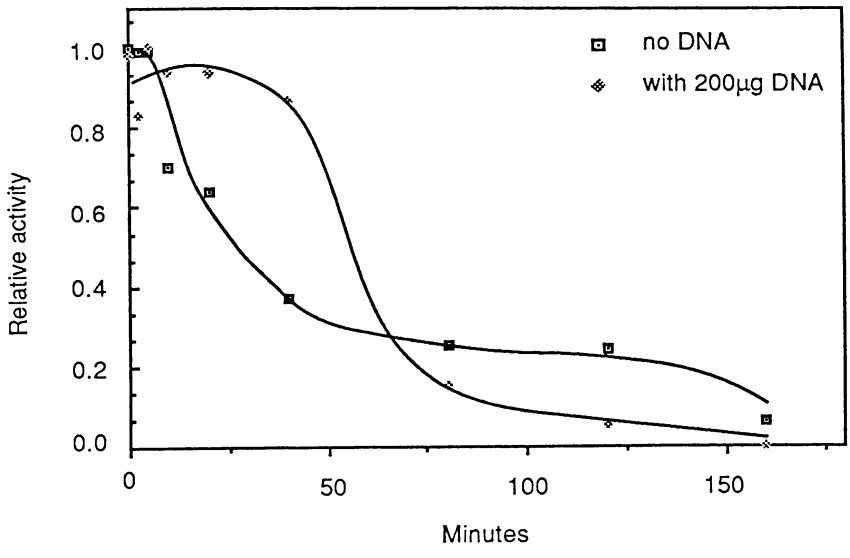


Figure 5.6.10 *S. solfataricus* reverse gyrase thermostability. Thermostability was investigated at 75°C by incubation of reverse gyrase in 50ug/ml bovine serum albumin, 50mM Tris/HCl pH7.5, and 1mM spermidine, and in the absence or presence of 200ug/ml double-stranded calf thymus DNA. After incubation for varying lengths of time, samples were assayed for activity under standard conditions.

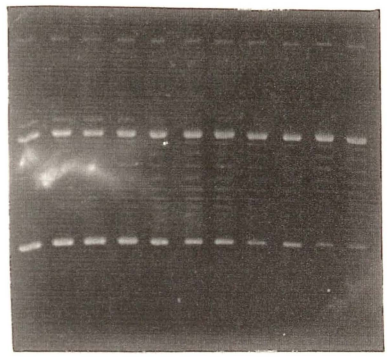


Figure 5.6.11 Agarose gel showing the effect of ATP on the activity of *S.solfataricus* reverse gyrase. The text of figure 5.6.9 describes the assay conditions for this experiment. Lanes, from left to right, were asayed at; 0, 1, 2, 4, 8, 16, 32, 80, 200, 500, and 1250uM ATP.

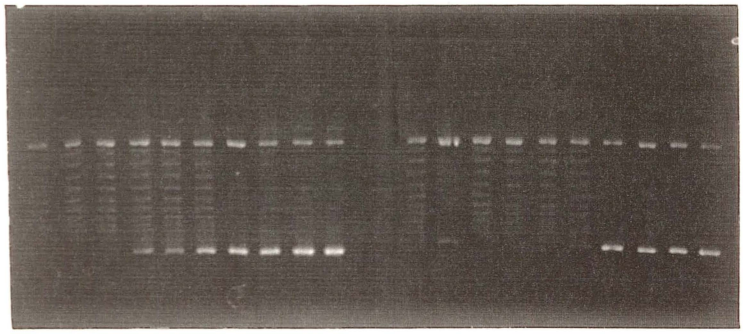


Figure 5.6.12 Agarose gel showing the thermostability of *S. solfataricus* reverse gyrase. Reverse gyrase was incubated for varying lengths of time and assayed for activity under standard assay conditions. Gel A shows denaturation of reverse gyrase in the absence of DNA while gel B shows denaturation of reverse gyrase in the presence of 200ug/ml double-standard calf thymus DNA. For both gels the lanes, from left to right, show reverse gyrase incubated at 75°C for; 0, 2.5, 5, 10, 20, 40, 80, 120, 160, and 240min.

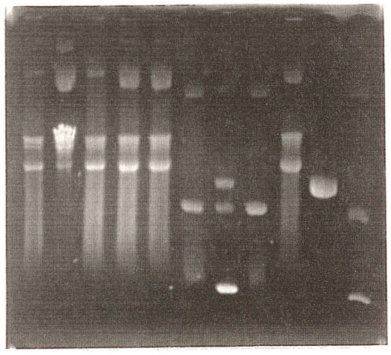


Figure 5.6.13 *S. solfataricus* reverse gyrase and *Bacillus caldovelox* topoisomerase activity on knotted P4 DNA. Reverse gyrase and *B. caldovelox* topoisomerase were assayed each under their standard assay conditions, and with pBR322 or knotted P4 DNA as the substrate. Lanes, from left to right, show; (1) knotted P4 DNA in reverse gyrase assay mix (not heated), (2) knotted P4 DNA in reverse gyrase assay mix (heated 80°C, 10min), (3) Ligated knotted P4 DNA in reverse gyrase assay mix (not heated), (4) Ligated knotted P4 DNA in reverse gyrase assay mix (heated 80°C, 10min), (5) Ligated knotted P4 DNA incubated with reverse gyrase (80°C, 10min) in reverse gyrase assay mix, (6) pBR322 incubated with reverse gyrase (80°C, 10min) in reverse gyrase assay mix, (7) pBR322 in reverse gyrase assay mix (80°C, 10min), (8) pBR322 incubated with *B. caldovelox* topoisomerase (70°C, 10min) in *B. caldovelox* topoisomerase assay mix, (9) Ligated knotted P4 DNA incubated with *B. caldovelox* topoisomerase (70°C, 10min) in *B. caldovelox* topoisomerase assay mix, (10) Ligated knotted P4 DNA cut with Eco R1.

5.6.6 The molecular weight of *S. solfataricus* reverse gyrase

The molecular weight of *S. solfataricus* reverse gyrase from purification scheme II was found to be 120,200Da using gel permeation FPLC (figure 5.4.4) and 122,500Da under denaturing conditions using SDS-PAGE (figure 5.4.5). These figures agree remarkably well with each other and also with the determined molecular weight of AN1 reverse gyrase (120kDa). They further suggest that reverse gyrase is active as a monomer and consists of a single polypeptide of about 120kDa. This conclusion agrees with that found by Nadal *et al.*, 1988 and Nakasu and Kikuchi, 1985 for their investigations on reverse gyrase structure.

5.6.7 Reverse gyrase activity on knotted P4 DNA

Knotted P4 DNA was used to indicate whether topoisomerase activity is of type I (single-stranded DNA nick) or type II (double-stranded DNA nick). Unknotting P4 duplex DNA requires the transient breakage and rejoining of double-stranded DNA which is specific to the type II enzyme. Knotted P4 DNA is not covalently closed and at high temperatures unknotting of the DNA occurs by disruption to hydrogen bonding at cohesive ends (Goto and Wang, 1982). The nicks at cohesive ends of the DNA were therefore sealed by T4 ligase before topoisomerase assays involving high temperatures were undertaken.

The result of an experiment to determine the effect of reverse gyrase and a *Bacillus* topoisomerase on ligated knotted P4 DNA is shown in figure 5.6.13. This figure shows unknotting of P4 DNA at 80°C and through the action of an endonuclease (Eco R1). However both reverse gyrase and the *Bacillus* topoisomerase exhibited no activity on this DNA substrate despite being active on negatively supercoiled pBR322. This leads to the conclusion that both are type I enzymes; however a positive control (type II topoisomerase) was not available during the course of this experiment which casts some doubt on the result.

5.6.8 Competition experiments with single-stranded M13 DNA

Single-stranded DNA is a potent inhibitor of relaxation reactions of all type I enzymes (Vosberg, 1985) but eubacterial type II topoisomerases are not inhibited (Srivenugopal *et al.*, 1984). When reverse gyrase was assayed under standard conditions with 0.5ug pBR322 and

varying amounts of single-stranded M13 DNA some slight inhibition was noticed (figure 5.6.14) but this was minimal when compared to that reported for *E. coli* type I topoisomerase (Srivenugopal *et al.*, 1984) or *Alcaligenes eutrophus* type I topoisomerase (Andera and Mikulik, 1990).

Inhibition of reverse gyrase activity by ssDNA has also been reported by Shibata *et al.*, 1987 (for *S. acidocaldarius*) and by Slesarev and Kozyavkin, 1990 (for *D. amylolyticus*) who also observed a small inhibitory effect with ssM13 DNA (at a molar ratio of 0.1 single- to double-stranded DNA). Slesarev and Kozyavkin, 1990 have pointed out that this inhibitory effect may be due to competition of the relaxation assay (which uses negatively supercoiled DNA) by the open circular plasmid DNA also found in the assay mix. At elevated temperatures open circular DNA would contain an increasing amount of single-stranded fluctuational openings to which reverse gyrase could bind. Based on the observed inhibition of the reverse gyrase enzyme by ssDNA, reverse gyrase may require a single-stranded or melted DNA region to bind before catalytic activity can occur. This is a feature which is also shared by the eubacterial type I topoisomerases.

An alternative explanation for the small effect of ssM13 DNA on reverse gyrase activity is that this enzyme, which preferentially binds to ssDNA (Slesarev and Kozyavkin, 1990), may also bind to dsDNA albeit with lower affinity. Such a possibility would not be surprising considering that reverse gyrase acts to positively supercoil DNA, an action that reduces or eliminates single-stranded regions, and it presumably remains attached while doing so.

5.6.9 The isoelectric point of *S. solfataricus* reverse gyrase

The isoelectric point was determined on samples of *S. solfataricus* reverse gyrase from both purification schemes. The isoelectric point of reverse gyrase purified from scheme I was found to be pI5.2 (figure 5.5.2) with activity being found in the main band and no activity found outside this band. It was postulated that this doublet band is composed of four major polypeptides, one or all of which are thought to be active proteolytic fragments of the reverse gyrase enzyme (section 5.5). It was surprising however that all four polypeptides showed the same isoelectric focusing properties and this could suggest that these polypeptides originated from a common origin, that is from a larger polypeptide.

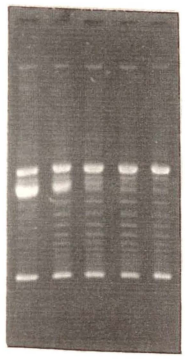


Figure 5.6.14 Inhibition of *S. solfataricus* reverse gyrase activity by single-stranded M13 DNA. Reverse gyrase was assayed under standard conditions with the inclusion of varying quantities of ssM13 DNA. Lanes, from left to right, were assayed with 0.5ug pBR322 and; 0.4, 0.125, 0.04, 0.0125, and 0ug ssM13 DNA. The molar ratios of ssDNA to dsDNA in the lanes were, from left to right, 1, 0.3, 0.1, 0.03, and 0.

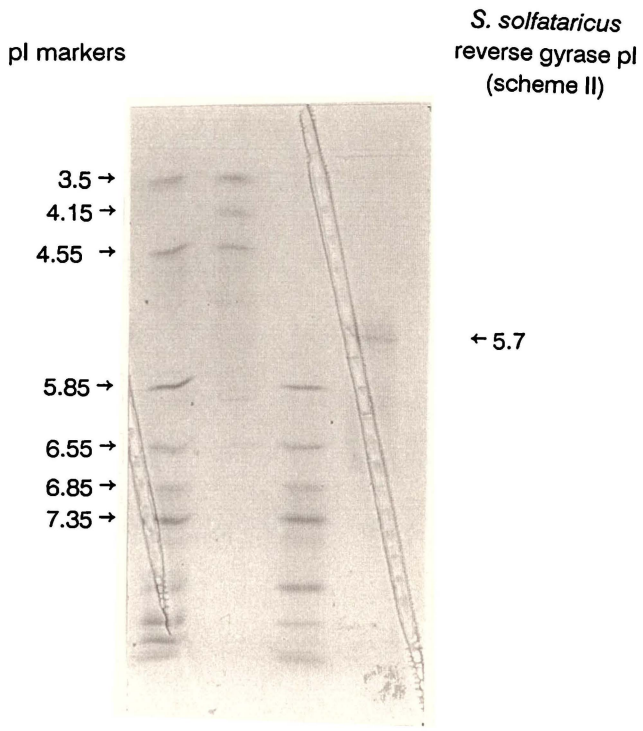


Figure 5.6.15 Isoelectric focusing of purified *S. solfataricus* reverse gyrase from Scheme II. Isoelectric focusing standards and the sample were run on a PhastGel IEF 3-9 using the procedures developed for isoelectric focusing with the PhastSystem (PhastSystem Operations Manual, Pharmacia). Lanes, from left to right, show; Pharmacia gold standards (pI3-10), Pharmacia red standards (pI2.5-6.5), Pharmacia blue standards (pI6-10), and purified reverse gyrase sample. The diagonal mark is the unfortunate result of drying the gel upside down. This does not comprimise the result in any way.

The isoelectric point of reverse gyrase purified by scheme II was determined to be 5.7 on a pH3-9 PhastGel using the PhastSystem equipment, (figure 5.6.15). This was the major band seen on the gel and furthermore was shown to contain active reverse gyrase in the following manner. A second lane was run with another reverse gyrase sample and the lane cut from the gel and sectioned into fractions. Each fraction, complete with plastic backing sheet, was added to a tube containing reverse gyrase assay mix and, after centrifugation at 10,000g for 5min, assayed under standard conditions for activity. Reverse gyrase activity was only seen in that fraction corresponding to the major protein band observable on the gel.

It was concluded that the 120kDa reverse gyrase from *S. solfataricus* had a pI of 5.7 and this is in accordance to the pI found for reverse gyrase from *S. acidocaldarius* by Nadal *et al.*, 1988 which was 5.6-5.9.

5.7 Discussion

The reverse gyrase from *S. solfataricus* was examined as this organism could be grown in large quantities and the quantity of purified reverse gyrase obtained should be sufficient to carry out N terminal amino acid sequencing and enzyme characterization.

The purification of reverse gyrase using the protocol in scheme I was not successful to the extent that no polypeptide of a molecular weight comparable to that expected for reverse gyrase from previous reports was isolated. The final active reverse gyrase preparation consisted of four major polypeptide bands of 38,900, 33,700, 28,200, and 24,800Da as determined by SDS-PAGE (figure 5.3.6). Although the possibility cannot be excluded that the observed activity in this fraction is due to unobservable amounts of reverse gyrase with a high molecular weight, the total absence of such a band even on a silver stained gel suggests that one or more of the low molecular weight polypeptides was exhibiting activity. Furthermore it may be possible that under non-denaturing conditions two or more of the polypeptides could combine to form an active fragment. Proteolytic digestion of topoisomerases during purification is not uncommon (Liu, 1983; Osheroff, 1989) and the fragments produced have often been noted to contain topoisomerase activity (Liu, 1983; Osheroff, 1989). It is interesting to note that the four major polypeptides isolated by scheme I have a combined molecular weight of 125.6kDa which leads to

the speculation that these fragments originate from a single polypeptide of this molecular weight (which is a similar size to previously reported reverse gyrase enzymes).

A second attempt at purifying reverse gyrase using scheme II was successful with a major polypeptide of about 120kDa being isolated which displayed reverse gyrase activity. An attempt to sequence the N terminus of this polypeptide did not succeed due to blocking of the N terminal amino acid, however characterization of the enzyme was achieved.

Characterization of reverse gyrase from *S. solfataricus* showed that this enzyme is indistinguishable to that found from other sulphur metabolizing archaebacterial species (compare with table 4.4.1 and see section 4.4). The characteristics of *S. solfataricus* reverse gyrase are summarized below:

(a) Maximum activity at a temperature of 80°C which was also found for the reverse gyrase of *S. acidocaldarius* (Kikuchi *et al.*, 1986). However the temperature optimum for reverse gyrase from AN1 (this thesis, chapter 4) and *D. amylolyticus* (Slesarev, 1988) was found to be 85-90°C and >100°C respectively. It is not known whether these differences in temperature optimum are significant or are due to variations in enzyme preparation, concentration, or assay conditions.

(b) A pH optimum of 6.3-6.4 which corresponds with the pH optimum found for AN1 reverse gyrase (6.3-6.5).

(c) A salt (NaCl or KCl) requirement of ~200mM for optimum relaxation of negatively supercoiled DNA.

(d) A magnesium requirement of ~10mM for optimum activity.

(e) An ATP requirement for activity.

(f) Knotted P4 DNA is not cleaved by reverse gyrase which makes this enzyme a type I topoisomerase.

(g) ssDNA competes to some extent with the negatively supercoiled plasmid used in the assay. This feature had been previously noted by Shibata *et al.*, 1987 and, Slesarev and Kozyavkin, 1990 with reverse gyrase from other archaebacterial species.

(h) A pI of 5.7 for reverse gyrase from *S. solfataricus*. This is in agreement with the isoelectric point of 5.6-5.9 for reverse gyrase from *S. acidocaldarius* (Nadal *et al.*, 1988).

CHAPTER SIX

PARTIAL PURIFICATION AND CHARACTERIZATION OF DNA TOPOISOMERASE I FROM BACILLUS CALDOVELOX

6.1 Introduction

The topoisomerase from this organism was examined in detail; first because topoisomerase activity was noticed in crude cell extracts of this organism during a general screen for topoisomerases (see chapter three), second because extracts from this organism were available in large quantities as a by-product of another project, and last because only one thermophilic eubacterial topoisomerase had been previously characterized (Yamamoto *et al.*, 1985) and a comparison between this and other eubacterial topoisomerases would be of interest.

The type I topoisomerase which Yamamoto *et al.* isolated from *Bacillus stearothermophilus* (optimal growth temperature, 65°C) was purified by ammonium sulphate precipitation and column chromatography using phosphocellulose, DEAE-cellulose, and Heparin Agarose. Despite the addition of PMSF, which acts to prevent proteolysis, three separate enzyme activities were obtained after Heparin Agarose chromatography. One of these fractions (150kDa) appeared to be a classical eubacterial topoisomerase I requiring only Mg^{2+} for full activity whereas the other two fractions (110 and 100kDa, 80kDa) required 150-200mM NaCl or KCl in addition to Mg^{2+} for activity. All three fractions from *B. stearothermophilus* exhibited maximum activity at 65-70°C.

6.2 Purification of *B. caldovelox* topoisomerase

Section 2.10 describes the procedures used in this purification. The cells used in this purification were lysed chemically. A low initial pH of 5.5 resulted in more complete and reproducable lysis than was found at higher pH values (Patchett, 1988) and the final pH of the

lysate supernatant was 6.5. The inclusion of Triton X-100 in the lysis buffer decreased the volume of the pelleted cell debris. Anion exchange (MonoQ) FPLC removed 85% of the protein whilst the remaining 15% contained the majority of activity as estimated from the purification profile. Activity was not eluted while washing with the starting buffer but was eluted mid-way through the salt gradient (0.5-0.65M NaCl). Pooled fractions containing peak topoisomerase activity displayed two major bands upon SDS-PAGE (figure 6.2.1) with molecular weights of 92.6 and 62.7kDa. The active band or bands in the pooled fractions was not determined.

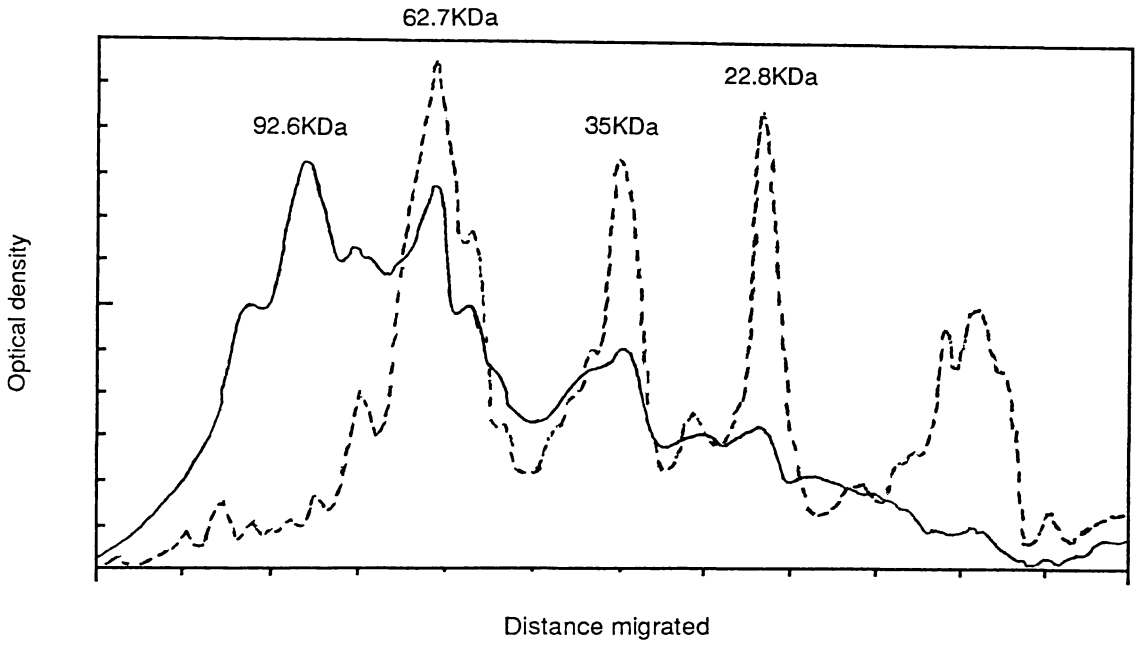


Figure 6.2.1 Scanning laser densitometry of SDS-PAGE (PhastGel 10-15% gradient) showing two lanes. The continuous line shows a lane run with pooled fractions containing topoisomerase activity after MonoQ chromatography. The discontinuous line shows a lane run with the protein sample loaded onto the MonoQ column. The 92.6kDa protein has been considerably purified by MonoQ chromatography.

6.3 Characterization of the *B. caldovelox* topoisomerase

6.3.1 pH and temperature optimum

The pH optima of *B. caldovelox* topoisomerase was determined at 75°C in reverse gyrase assay buffer as topoisomerase activity was originally observed under these conditions. Initially a variety of buffers were used in order to assay for activity over a wide pH range

(MOPS/NaOH, MES/NaOH, Na Acetate/Acetic acid), however the sulfonic acid based buffers, such as MOPS and MES, appeared to inhibit enzyme acitivity and a single buffering system using bis-Tris Propane/HCl was used, which owing to its combination of two pKa's at 6.8 and 9.0, buffers over a wide pH range. An increase of 1°C decreases the pKa of this buffer by 0.016 (a pKa change of -0.8 from 20 to 70°C) and this was compensated for in the preparation of bis-Tris Propane/HCl buffers at room temperature.

Using the reverse gyrase assay constituents and bis-Tris Propane/HCl (50mM) as the buffer a broad pH range for optimal activity was seen centred at pH8.0 (figure 6.3.1.). Little activity could be discerned below pH7.0 or above 9.0.

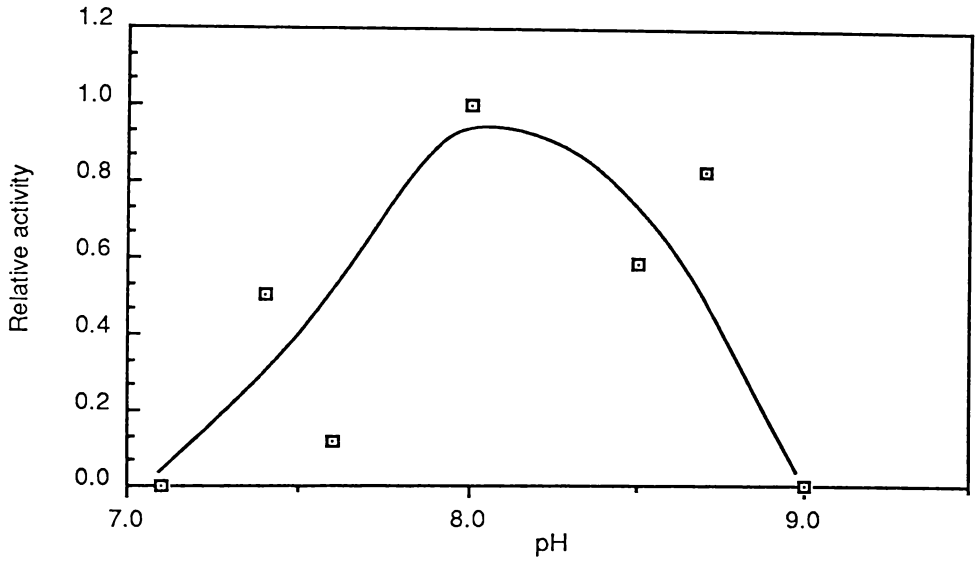


Figure 6.3.1 pH optimum of *B. caldovelox* topoisomerase. Assays were performed at 75°C under reverse gyrase assay conditions. 50mM bis-Tris Propane/HCl,adjusted to compensate for temperature effects, was used as the buffer over the whole pH range shown.

The optimum assay temperature was determined under reverse gyrase assay conditions except that the buffering agent was changed to bis-Tris Propane/HCl (50mM). The pH of this buffer was increased as the assay temperature was increased in order to compensate for the reduction of pH using bis-Tris Propane with increasing temperature. The final pH of the assay was always kept between 7.9-8.2. Activity of *B. caldovelox* topoisomerase was determined at temperatures ranging from 20-100°C and the resultant dependence of activity on temperature is shown in figures 6.3.2 and 6.3.5. Little activity could be detected below 60°C and the enzyme

was optimally active at a temperature of 65-70°C. Little activity could be discerned at temperatures of over 80°C. The temperature dependence of this topoisomerase corresponds to the growth temperature of *B. caldovelox* and is the same as the topoisomerase fractions found in *B. stearothermophilus* by Yamomoto *et al.*, 1985. The isolation of topoisomerases with similar temperature requirements from two species of thermophilic *Bacillus* was perhaps predictable given the essential nature of this enzyme. It would seem likely that these topoisomerases represent a subset of the normal eubacterial topoisomerase I which are adapted to acting at high temperatures. It would be of interest to see if phylogenetically distinct thermophilic eubacteria contained topoisomerases with similar characteristics.

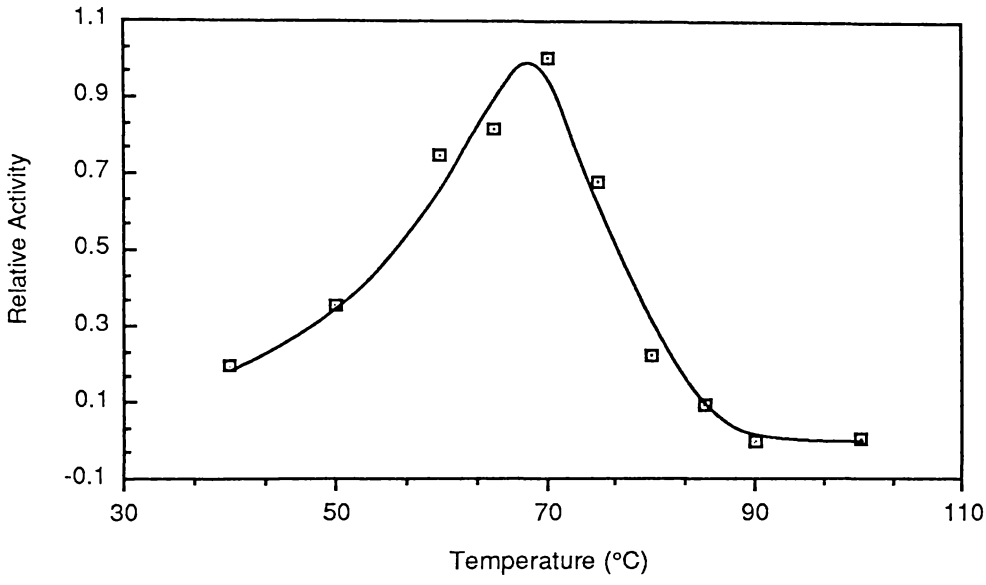


Figure 6.3.2 The effect of temperature on *B. caldovelox* topoisomerase activity. Reverse gyrase assay conditions, with 50mM bis-Tris Propane/HCl as the buffer, was used for these experiments.

6.3.2 Magnesium and Salt requirements

Optimal activity of the *B. caldovelox* topoisomerase was found at a magnesium concentration of 7mM, shown in figures 6.3.3 and 6.3.6. No activity could be detected in the absence of magnesium, however even low concentrations of this divalent cation (1mM) produced reasonable levels of activity. Magnesium concentrations of 60mM or greater resulted in no activity.

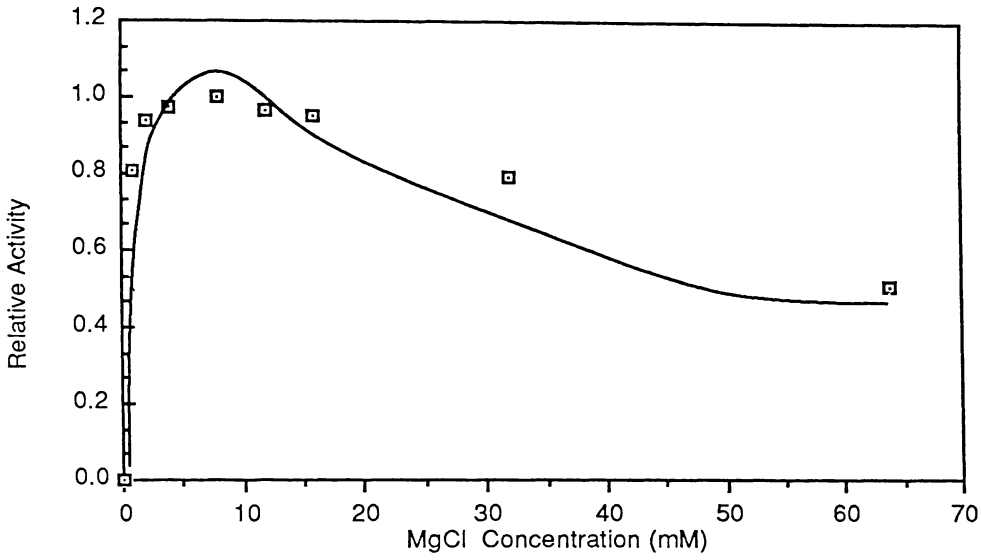


Figure 6.3.3 Effect of varying concentrations of magnesium on *B. caldovelox* topoisomerase activity. *B. caldovelox* topoisomerase was assayed at 70°C under reverse gyrase assay conditions with 50mM bis-Tris Propane/HCl pH8.0 (70°C) as the buffer and in the presence of varying concentrations of Mg^{2+}.

Neither NaCl or KCl was required for full activity and increasing concentrations of these ions linearly decreased activity until a concentration of 400mM was reached at which point activity was minimal (see figures 6.3.4 and 6.3.7). An increase in concentration of KCl was less detrimental to topoisomerase activity than NaCl.

The dependence of *B. caldovelox* topoisomerase on 5mM magnesium and its independence of a NaCl or KCl requirement would seem to indicate that this enzyme is a classical eubacterial type I topoisomerase. Yamamoto *et al.*, 1985 found an enzyme with similar properties in one of their fractions (fraction c) from *B. stearothermophilus*, however the other two topoisomerase active fractions which they isolated showed a typically non-eubacterial dependence upon monovalent cations for activity. Topoisomerases displaying such characteristics were not shown to be present in this *B. caldovelox* sample, however if they were present then their activity may have been masked by the typically eubacterial like topoisomerase.

It was decided to use 100mM NaCl in subsequent assays of *B. caldovelox* topoisomerase as at this concentration of salt the enzyme worked in a distributive manner which was easier to quantify than the processive mode utilised when salt was absent.

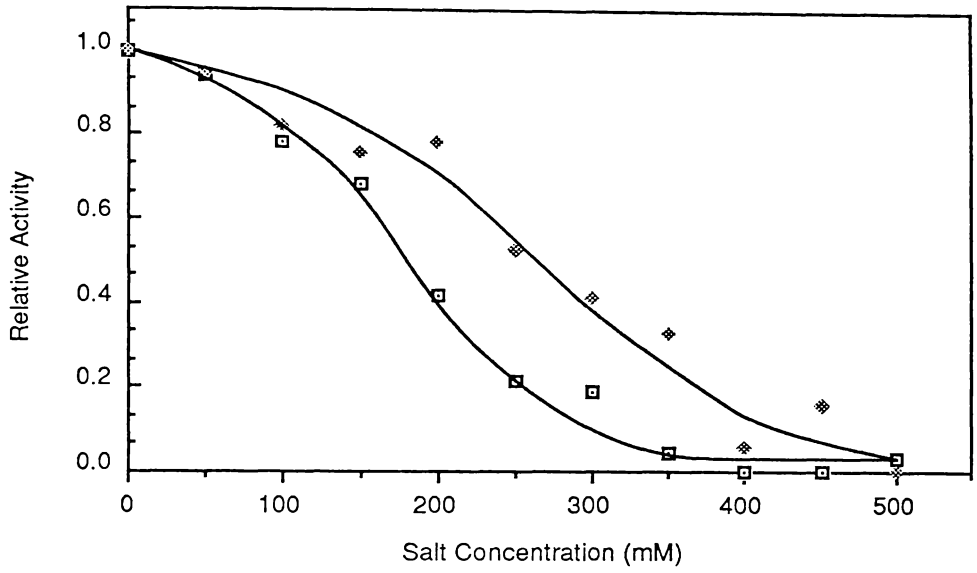


Figure 6.3.4 The effect of varying salt concentrations on the activity of *B. caldovelox* topoisomerase. Square data points show relative activity in the presence of NaCl while diamond data points show relative activity in the presence of KCl. *B. caldovelox* topoisomerase was assayed at 70°C under standard reverse gyrase assay conditions with 50mM bis-Tris Propane/HCl pH8.0 (70°C) as the buffer.

6.3.3 The effect of ATP on activity

Figure 6.3.9 shows that the *B. caldovelox* topoisomerase does not require ATP for activity. This places this enzyme with the eubacterial topoisomerase type I enzymes which do not require ATP for activity. The *B. caldovelox* topoisomerase appears to be slightly inhibited by concentrations of ATP of 500uM or higher. This inhibition has not been reported for other eubacterial type I topoisomerases where ATP apparently has no effect even at concentrations of up to 4mM (Andera and Mikulik, 1990; Srivenugopal *et al.*, 1984).

6.3.4. Thermostability of the *B. caldovelox* topoisomerase at 75°C

Thermostability was assayed by incubating the topoisomerase in 10mM Tris/HCl pH7.5, 100mM NaCl, and 10% glycerol at 75°C for varying lengths of time. Topoisomerase activity was then assayed as usual at 70°C for 10min. Under these conditions topoisomerase activity initially decreased logarithmically before levelling off (see figure 6.3.8). A half life of approximately 4.5min was determined for this enzyme at 75°C under the previously mentioned conditions.

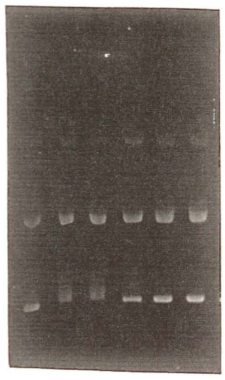


Figure 6.3.5 Agarose gel showing the effect of temperature on *B. caldovelox* topoisomerase activity. Reverse gyrase assay conditions, with 50mM bis-Tris Propane/HCl as the buffer, were used for these experiments. Lanes, from left to right, are assayed at; 60, 65, 70, 75, 80, and 85°C.

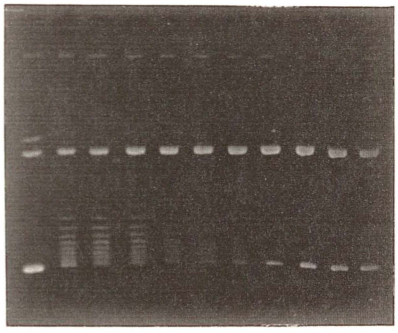


Figure 6.3.6 Agarose gel showing the effect of Mg^{2+} on the activity of *B. caldovelox* topoisomerase. The text of fig 6.3.4 describes the assay conditions of this experiment. Lanes, from left to right, are assayed at; 0, 1, 2, 4, 8, 12, 16, 32, 64, 128, and 256mM $MgCl_2$.

A B

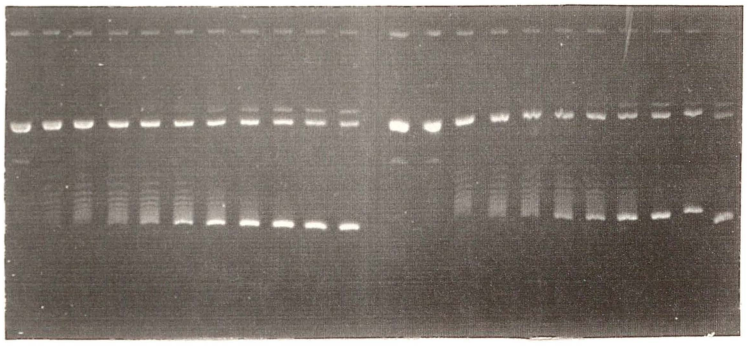


Figure 6.3.7 Agarose gel showing the effect of salt on the activity of *B. caldovelox*topoisomerase. The text of figure 6.3.6 describes the assay conditions for this experiment. Gel A shows the effect of NaCl on activity, gel B shows the effect of KCl on activity. For both gels the lanes, from left to right, are assayed at; 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500mM salt.

It would seem likely that cytoplasmic components would stabilize topoisomerases to a greater extent then was found in the *in vitro* assay, for example it was determined that the addition of dsDNA appears to increase the thermostability of this enzyme although the extent of this stabilization was not quantified.

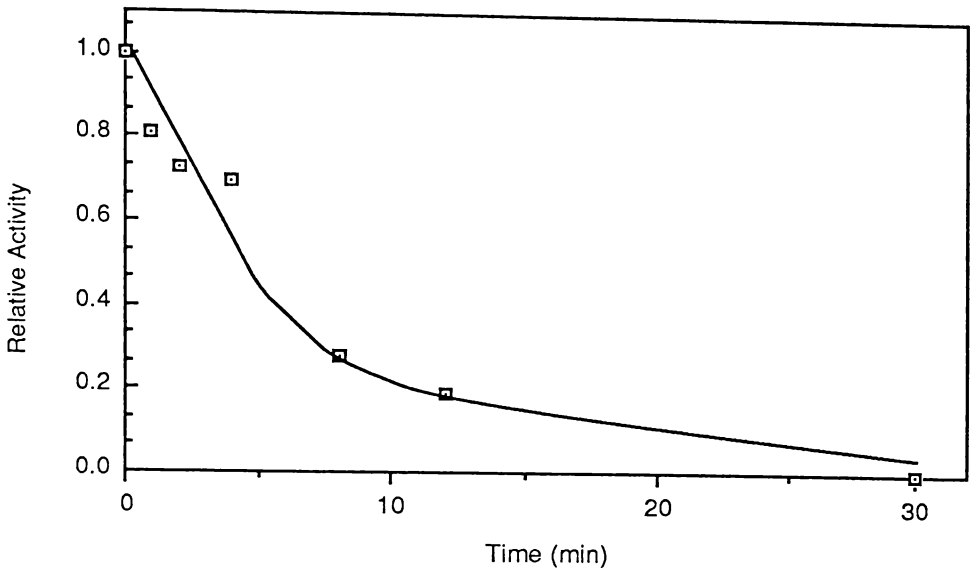


Figure 6.3.8 *B. caldovelox* topoisomerase thermostability. Thermostability of *B. caldovelox* topoisomerase was determined by incubating at 75°C in 10mM Tris/HCl pH7.5, 100mM NaCl, and 10% glycerol for varying lengths of time. Samples were assayed for activity in *B. caldovelox* assay buffer (50mM bis-Tris Propane pH8.0 (70°C), 100mM NaCl, 5mM $MgCl_2$, 1mM DTT, and 1mM spermidine) for 10min at 70°C.

6.3.5. Molecular weight of the *B. caldovelox* topoisomerase

The molecular weight of this topoisomerase was determined by non-denaturing analytical gel filtration on a TSK-gel G3000SW HPLC column, to be 87-93kDa. Only one peak of activity was seen during this experiment which would suggest that proteolytic digestion of this enzyme had not occurred to yield catalytically active fragments of lower molecular weights, a problem which has been experienced in many topoisomerase purifications (Vosberg, 1985; Yamamoto *et al.*, 1985). The same sample on denaturing SDS PAGE (figure 6.2.1) displayed two major protein bands. The largest of these bands had a molecular weight of 92.6kDa, similar to the molecular weight determined by analytical gel filtration. This band showed considerable purification upon MonoQ FPLC chromotography for topoisomerase. If one accepts that this band is indicative of the topoisomerase protein then this would suggest that this enzyme is a

monomer of approximately 93kDa. This appears to be reasonable in the light of the structure and molecular weight determined for other eubacterial type I topoisomerases which are also monomeric enzymes with molecular weights of around 100kDa (Andera and Mikulik 1990; Srivenugopal *et al.*, 1984).

6.3.6. Inhibition by single-stranded DNA

The *Bacillus* topoisomerase is inactivated by ssDNA as determined by the inhibition of relaxation of negatively supercoiled pBR322 by addition of single-stranded M13 DNA (figures 6.3.10 and 6.3.12). Each track in figure 6.3.10 contains 0.5ug of pBR322, 60% of which is in the nicked circular form. This DNA may act as a competitive inhibitor of the topoisomerase reaction in addition to ssM13 as at elevated temperatures single-stranded regions are more likely to be present (Slesarev and Kozyavkin, 1990). Under the assay conditions utilised ssM13 DNA substantially retards the topoisomerase reaction at levels higher than 0.125ug M13 to 0.5ug pBR322 (a molar ratio of 0.3). Relaxation of pBR322 was totally inhibited when 0.4ug ssM13 was added to the assay system (a molar ratio of 1 molecule of ssM13 to 1 molecule of pBR322). In this respect the *B. caldovelox* topoisomerase appears to differ from the *Alcaligenes eutrophus* type I topoisomerase where a molar ratio of 1/50 ssDNA to dsDNA was effective in inhibiting topoisomerase activity (Andera and Mikulik, 1990). The apparent lower affinity of the *B. caldovelox* enzyme for M13 DNA may possibly be due to the aforesaid inhibitory activity of open circular DNA and also to a greater degree of fluctuational openings of the negatively supercoiled pBR322 substrate at the higher temperature in which the *Bacillus* topoisomerase is assayed (Depew and Wang, 1975), rather than to any intrinsic difference in enzyme mechanism or activity between the two topoisomerases.

6.3.7. *B. caldovelox* topoisomerase activity on knotted P4 DNA

The *B. caldovelox* topoisomerase was not active on ligated knotted P4 DNA under the assay conditions normally utilised for this enzyme (figure 5.6.13). This would suggest that this topoisomerase is a type I enzyme cutting only a single-strand of the DNA helix at any one time. This is supported by the observation that negatively supercoiled DNA changes in steps of 1 to

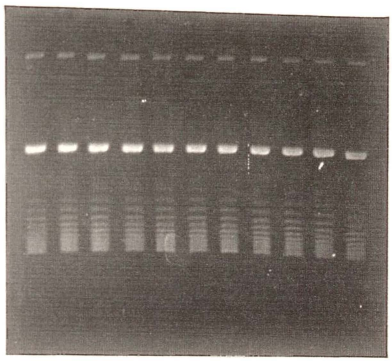


Figure 6.3.9 The effect of ATP on the activity of *B. caldovelox* topoisomerase. *B. caldovelox* topoisomerase was assayed in an assay solution containing 50mM bis-Tris Propane/HCL pH8.0 (70°C), 1mM spermidine, 5mM $MgCl_2$, 1mM DTT, and 100mM NaCl and in the presence of varying concentrations of ATP. Lanes, from left to right, are assayed at; 0, 1, 2, 4, 8, 16, 32, 80, 200, 500, and 1250uM ATP.

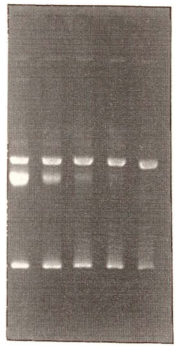


Figure 6.3.10 Agarose gel showing the effect of single-stranded M13 DNA on the activity of *B. caldovelox* topoisomerase. The conditions of assay are described in the text of figure 6.3.9. Lanes, from left to right, are assayed at; 0.4, 0.125, 0.04, 0.0125, and 0ug M13 DNA. Molar ratios of ssDNA to dsDNA in the lanes are, from left to right, 1, 0.3, 0.1, and 0.

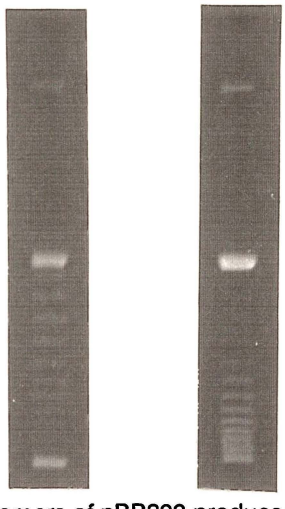


Figure 6.3.11 A comparison of the topoisomers of pBR322 produced by reverse gyrase (left hand lane) and *B. caldovelox* topoisomerae I (right hand lane). Both topoisomerases reduce the linking number of pBR322 in steps of one. For the *B. caldovelox* topoisomerase under distributive assay conditions the distribution of topoisomers is more negatively supercoiled than that found for reverse gyrase.

give topoisomers differing by one linking number. This gives identical topoisomers of plasmid DNA to that produced by the type I enzyme reverse gyrase (figure 6.3.11). Many of the other characteristics discussed in this chapter, ATP independence for example, reinforce the notion that this enzyme is a type I topoisomerase with most of the classical features which identify this particular group of enzymes.

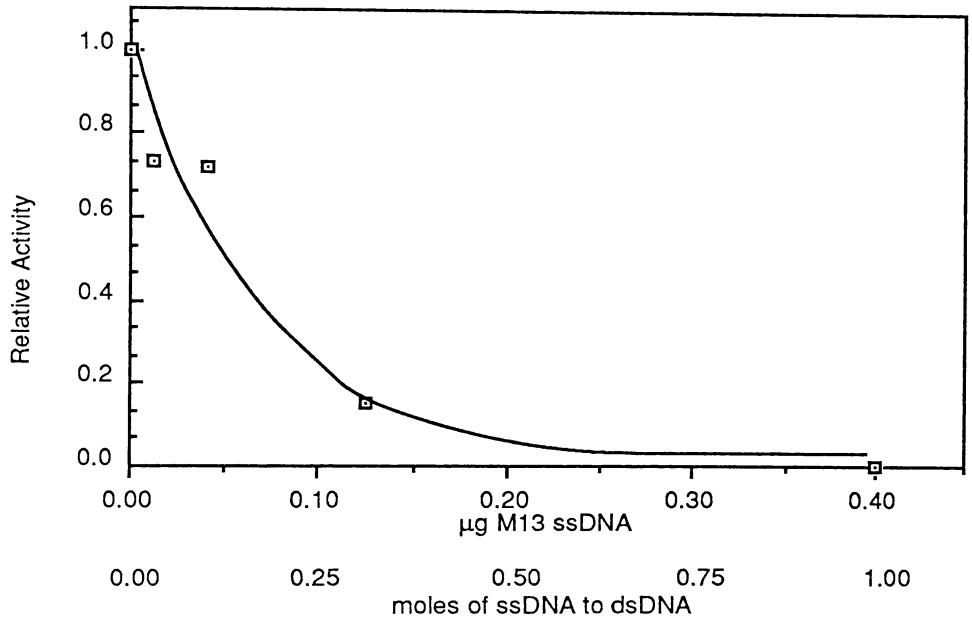


Figure 6.3.12 Inhibition of *B. caldovelox* topoisomerase activity by single-stranded M13 DNA. *B. caldovelox* topoisomerase was assayed in *B. caldovelox* assay buffer containing 0.5ug pBR322 and varying quantities of M13 DNA at 70°C for 10min.

6.4. Discussion

The topoisomerase I enzyme from *B. caldovelox* displays all of the standard attributes of a typical eubacterial type I enzyme with the exception of its requirement for a high temperature (65-70°C) for optimal activity. It is interesting that this particular enzyme can be easily assayed in crude cell lysates and is apparently the dominant topoisomerase under the assay conditions employed (which were equally suitable for the expression of both gyrase and type I activities). One could speculate that, similar to reverse gyrase, a potent relaxing topoisomerase is needed in these thermophilic organisms in order to balance the activity of the putative DNA gyrase so that the intracellular state of the DNA does not become negatively supercoiled to an extent that would result in excessive denaturation of the DNA helix.

CHAPTER SEVEN

ENDEAVOURS TO DETERMINE THE SUPERHELICAL DENSITY OF THE ARCHAEBACTERIAL GENOME

7.1 Introduction

Reverse gyrase is known to positively supercoil plasmid DNA *in vitro* but no studies of its effects on the archaebacterial chromosome have been published. However it has been demonstrated that the DNA of a virus like particle (SSV1) isolated from *Sulfolobus solfataricus* is mainly found in a positively supercoiled form (Nadal et al., 1986).

Given the predominance of positive supercoiling activity in crude extracts of sulphur metabolizing archaebacteria it would seem quite likely that their genomic DNA is positively supercoiled. If this is the case then these bacteria are unique in this characteristic as all other organisms contain negatively supercoiled DNA (Liu, 1987; Sinden et al 1980).

This section of research was aimed at determining the state of supercoiling of chromosomal DNA from two representative species of sulphur metabolizing archaebacteria (*Sulfolobus solfataricus* and AN1). The method utilised follows that used for *E. coli* (Worcel and Burgi, 1972) where the nucleoid was isolated and analysed on sucrose gradients with various concentrations of ethidium bromide. The superhelical density of the genomic DNA can be ascertained by determining the ethidium bromide concentration at which the nucleoids mobility is most retarded (Sloof *et al.*, 1983; Crawford and Waring, 1967) (see figure 7.1.1).

The use of ethidium bromide, which positively supercoils DNA, is useful only for the determination of negatively supercoiled genomes. Its effect on the mobility of positively supercoiled genomes would be expected to be minimal with only a slight increase in mobility occurring if the genome was not positively supercoiled to the maximum extent. To determine the density of positively supercoiled DNA, or genomes containing positively supercoiled DNA, a drug is needed which acts to negatively supercoil DNA. Netropsin, which binds to B-DNA within the

minor groove (Kopka *et al.*, 1985) is such a drug (Snounou and Malcolm, 1983). At the appropriate concentrations netropsin would give a similar result of nucleoid mobility versus drug concentration with positively supercoiled DNA as ethidium bromide gives with negatively supercoiled DNA.

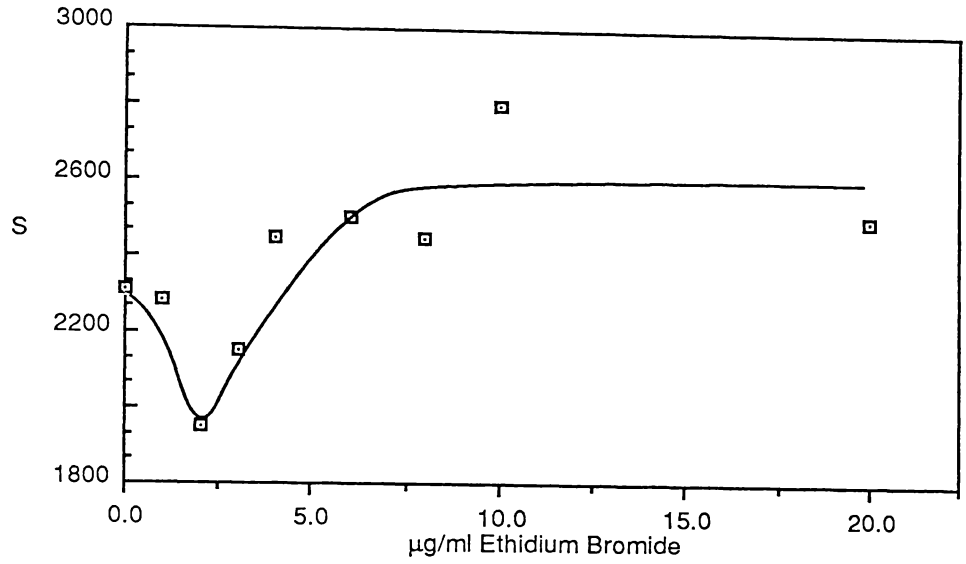


Figure 7.1.1 Determination of the superhelical density of *E. coli* genomic DNA. Data from Worcel and Burgi, 1972. The sedimentation of these nucleoids is maximally retarded at an ethidium bromide concentration of 2ug/ml. This equates to a superhelical density of -0.18 for this DNA (determined using the protocol in section 2.13.6).

The lysis conditions used in attempts to isolate nucleoids from *S. solfataricus* were obtained from two sources. Firstly a 2mM spermidine, 1% Nonidet P-40 lysis protocol was employed which had been reported in the isolation of nucleoids from *S. acidocaldarius* (Reddy and Suryanarayana, 1988) and secondly a 1M NaCl, 1% Bridj 58, 0.4% deoxycholate lysis protocol was employed which had been used to isolate nucleoids from *E. coli* (Stonington and Pettijohn, 1971; Worcel and Burgi, 1974) These procedures were varied in attempts to isolate nucleoids from *S. solfataricus*. This portion of research was hindered by the lack of a suitable method for determining whether nucleoids were produced under the various lysis conditions employed . Initial screening for the possible production of nucleoids was carried out by checking for the absence of viscosity in the preparation and the presence of nucleoid type structures, as seen for *E. coli* (figure 7.1.2), under the phase contrast microscope. If both the above conditions were realized then the preparation was incubated at a final concentration of 1% SDS at 70° for

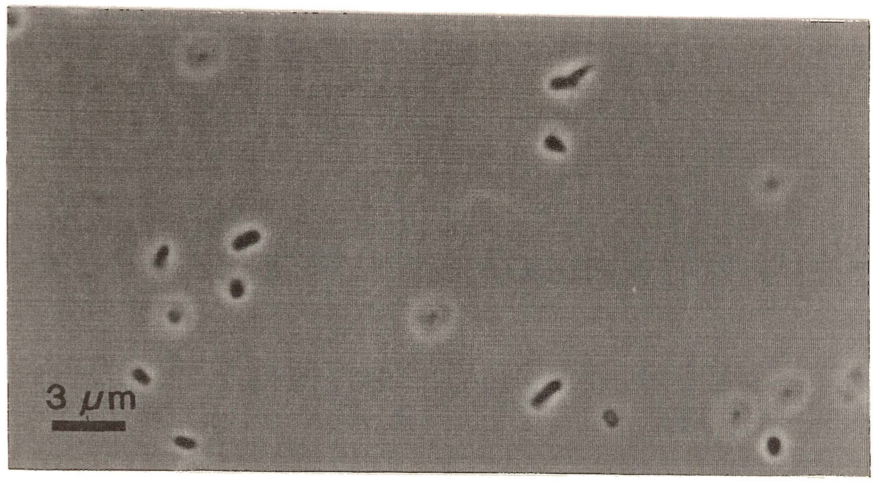


Figure 7.1.2 (a) *E. coli* cells (phase contrast microscopy). In focus cells are phase dark rods.

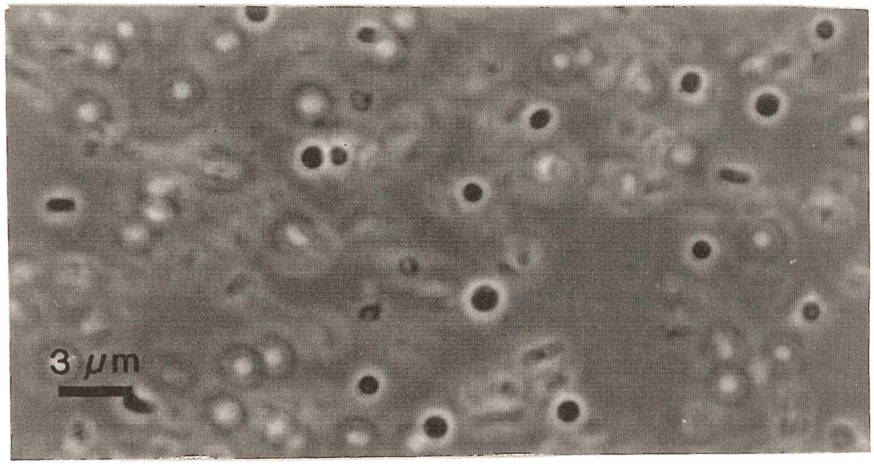


Figure 7.1.2 (b) *E. coli* spheroplasts (phase contrast microscopy). *E. coli* cells were partially digested with lysozyme so that both intact cells and sphaeroplasts can be seen. Spheroplasts appear as phase dark circular structures.

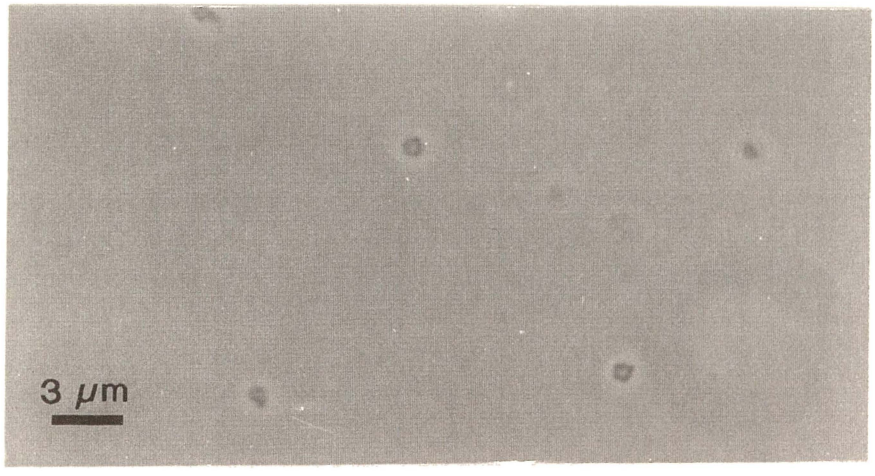


Figure 7.1.2 (c) *E. coli* nucleoids (phase contrast microscopy)..Nucleoids are very phase light structures and are pleomorphic but tending to be circular.

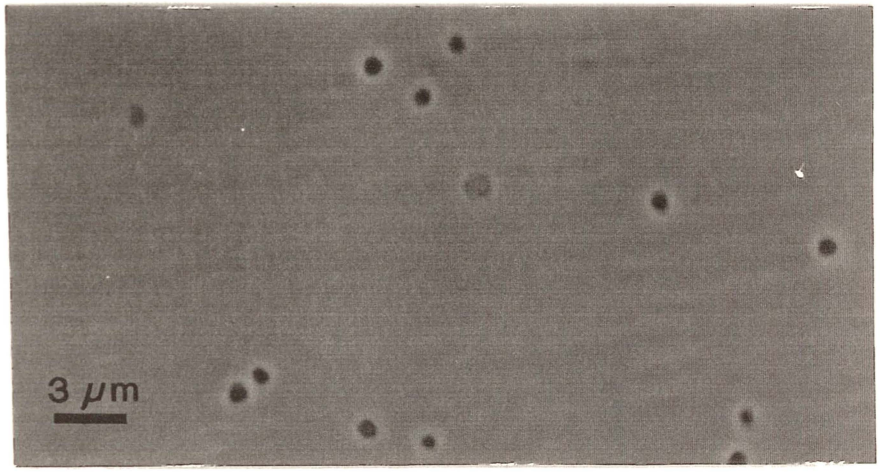


Figure 7.1.2 (d) *S. solfataricus* cells (phase contrast microscopy). In focus cells are lobed and phase dark often with a darker area at the extremity of a lobe.

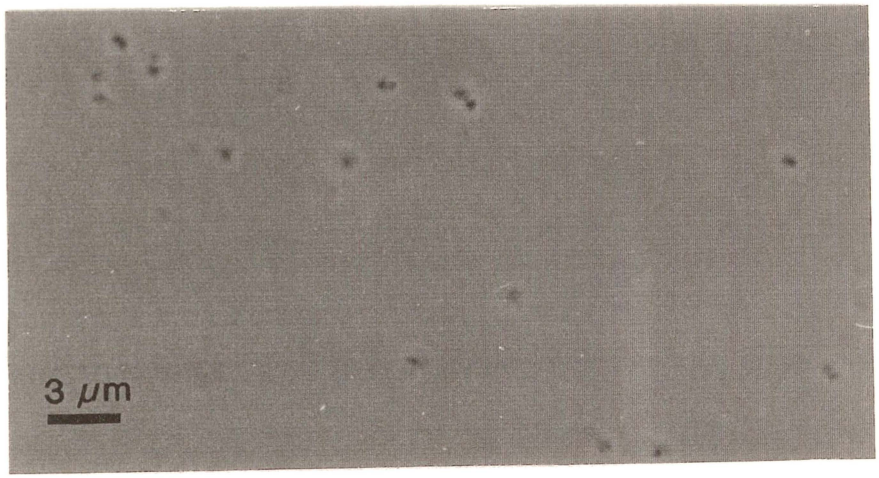


Figure 7.1.2 (e) *S. solfataricus* 'nucleoids' (phase contrast microscopy). In focus 'nucleoids' are phase light in comparison to cells though darker areas can be discerned.

5min. If the preparation became viscous then the nucleoid type structures (hereinafter called 'nucleoids') seen under phase contrast microscopy were, at least in part, composed of DNA.

The series of experiments in sections 7.4.3, 7.4.4, and 7.5.2 were run in order to observe whether a difference in mobility of the 'nucleoids' occurred at different concentrations of ethidium bromide or netropsin. If mobility change occurred than the existence of a true nucleoid could be inferred as only exposed supercoiled DNA structures would have their density and hence mobility affected by these drugs. The concentrations of ethidium bromide which were used in these experiments followed that used by previous researchers (Stonington and Pettijohn, 1971; Worcel and Burgi, 1974). However no papers on the use of netropsin to study supercoiling of nucleoids have been published. It was therefore important to determine the concentrations of netropsin that should be used in these studies as concentrations that were too low would have a minimal effect on supercoiling whereas concentrations above a certain value would be expected to exert maximal negative supercoiling. In both such cases it was likely that no changes in the mobility of positively supercoiled DNA would be observed.

To the best of my knowledge only one paper has described the use of netropsin in sucrose gradients (Wartell *et al.*, 1974). This paper compares the effects of ethidium bromide and netropsin on negatively supercoiled M13 RF1 DNA and concludes that, in contrast to ethidium bromide, netropsin has no effect on sedimentation of negatively supercoiled DNA. The concentrations of netropsin used in these studies were from 0-35.3uM with most data points in the range of 0-6.3uM. Using agarose gel electrophoresis Snounou and Malcolm, 1983 have examined the effect of netropsin on the supercoiling of plasmid DNA. Their results show that netropsin binds to DNA in a manner which causes negative supercoiling of closed circular DNA. By varying the concentrations of netropsin in the presence of excess topoisomerase I and then by removing both the enzyme and netropsin they discovered maximal positive supercoiling of the plasmid at 10uM netropsin. At 20uM netropsin no further supercoiling was observed.
From the results of these papers it was decided to use concentrations of 0-10uM netropsin in sucrose gradients bearing in mind that the association constant of netropsin for DNA would be lower than in these published experiments as a higher ionic concentration (1M NaCl) was being used (Wartell *et al.*, 1974). The extent to which this would effect the amount of netropsin bound

to DNA (and hence its supercoiling) was unknown. Figure 7.1.3 shows that a decrease in affinity of ethidium bromide (1.3 uM) to the netropsin-DNA complex occurs at increasing netropsin concentrations. This has already been reported (Wartell *et al.*, 1974). Furthermore this experiment confirms that netropsin binds to calf thymus DNA under both 0 and 1M salt concentrations.

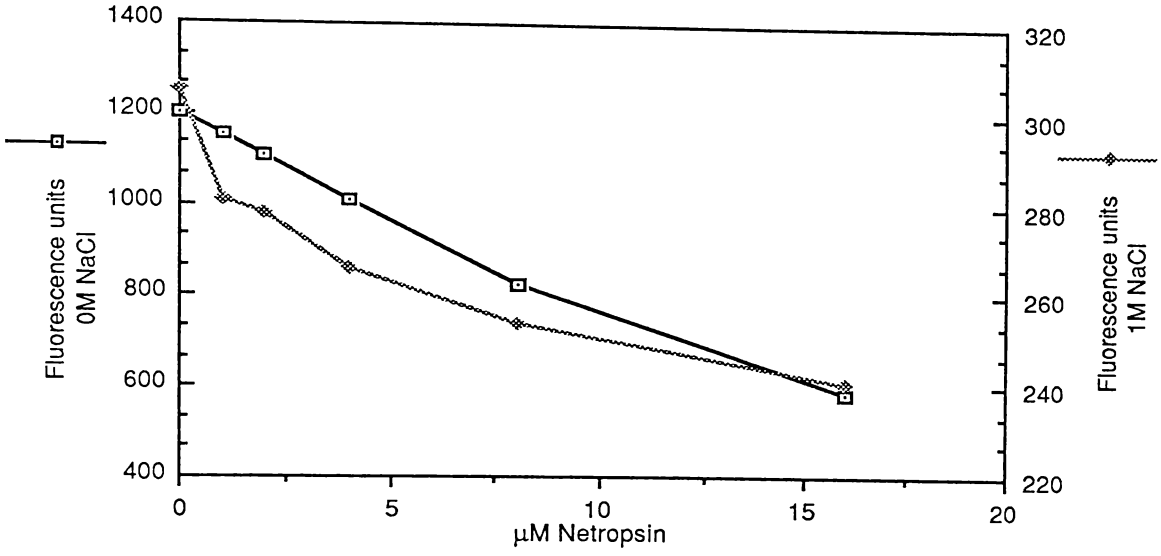


Figure 7.1.3 The effect of netropsin concentrations on ethidium bromide fluorescence of DNA under high and low salt conditions.

7.2 Sources of inaccuracy of results taken from sucrose gradient experiments

Sucrose gradients were prepared using the procedure described in section 2.13.4. The accuracy of results obtained from sucrose gradients was affected by:

(a) Reproducibility of sucrose gradient formation. Sucrose gradients were always prepared in the same manner (See section 2.9.3). The refractility of fractions taken from 3 gradients were determined and shown to be nearly identical (figure 2.13.1). This would seem to indicate that the reproducibility of sucrose gradients was not a major contributing factor in any inaccuracy of results from these experiments.

(b) Centrifugation conditions. This is another possible source of inaccuracy in results. However it is thought that this step produces few problems for accuracy due to the high reproducibility of runs on the Beckman T100 ultracentrifuge and more importantly the inclusion of

internal markers (phage T4 particles or nucleoid structures) in sucrose gradient experiments to determine that consistent results between runs were achieved.

(c) Fraction collection. Collection of fractions, the procedure of which is described in section 2.13.4, has the potential to be a major contributing factor in variation of fractions collected from different tubes. Variation in drop size or miscounting of drops are possible sources of error in this technique. However using the fraction collection technique outlined in section 2.13.4 a small variation of 0.5-1% in the total number of fractions collected per tube was observed under conditions in which surface active agents were absent (this equates to less than one half of a fraction if 40 fractions are collected). This accuracy is shown in figure 2.13.1 which reflects the reproducibility of both sucrose gradient formation and fraction collection.

(d) Drop size. Surface active agents had a large effect on drop size and hence numbers of fractions collected. This was especially noticeable between fraction collecting runs on preparative material, which contained high detergent concentrations from the lysis protocol, and analytical runs which were mostly free of detergent. This source of error was minimised by always washing the fraction collection apparatus between runs in order to remove contamination by surface active agents.

(e) Nucleic acid determination. The assay method used to determine DNA content is another potential source of error. Agarose gel electrophoresis and spectrofluorometry were used to identify nucleoid and nucleoid type material in sucrose gradient fractions (see section 2.13.5). Both of these methods gave similar results in the identification of DNA containing material (see figure 7.2.1). The use of agarose gels had the advantage of being able to unambiguously establish the presence of chromosomal DNA (figure 7.2.2) and chromosomal DNA only was determined using this technique whereas total nucleic acids were determined using spectrofluorometry. However the former method was cumbersome and less reproducible than using spectrofluorometry, therefore spectrofluorometry was used to determine DNA and DNA associated structures for all experiments involving sucrose gradients. Spectrofluorometry has been previously used to assay nucleoids from sucrose gradients (Morgan *et al.*, 1979).

(f) Quantitation of data. Data was standardised in the following manner. The data obtained was graphed and analysed on a MacIntosh SE computer using Cricket Graph©. The

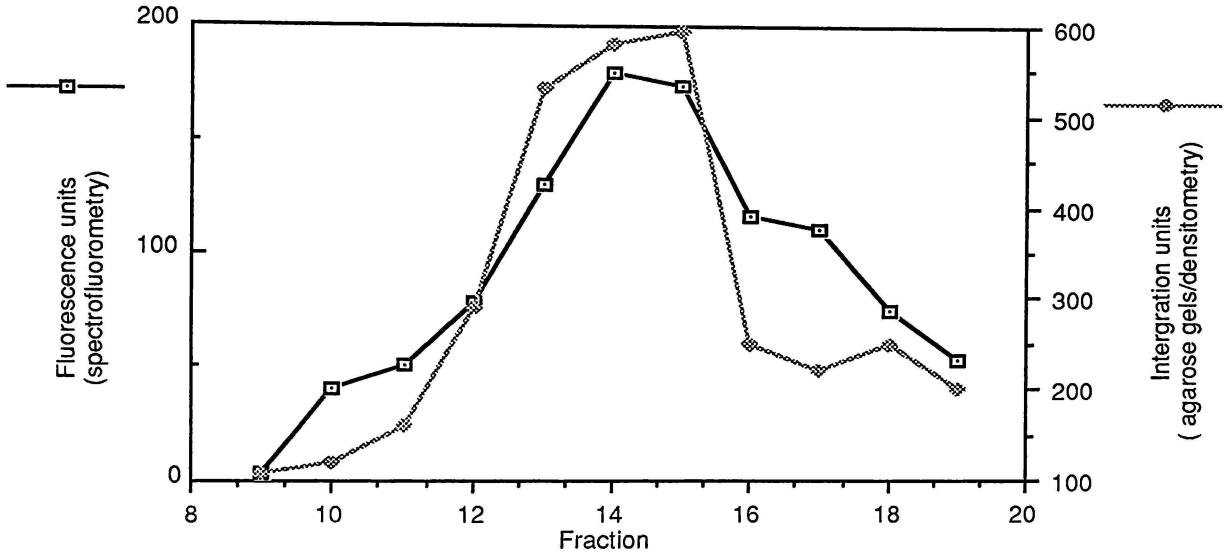


Figure 7.2.1 A comparison of spectrofluorometry and agarose gels/densitometry in determining nucleic acid content of 'nucleoids' run on sucrose gradients.

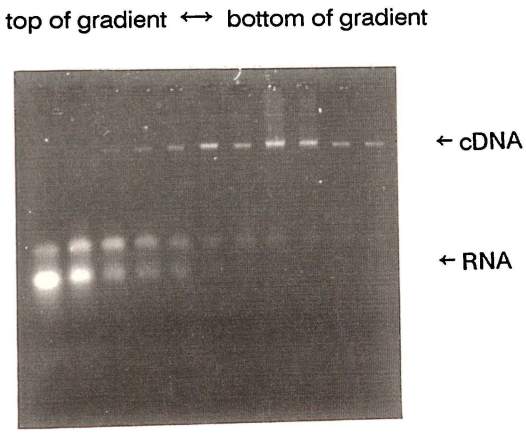


Figure 7.2.2 Agarose gel of fractions from a sucrose gradient run with an *E. coli* nucleoid preparation.

resulting nucleoid peak from analytical runs was expected to be normal in distribution and the centre of the peak was obtained using a fifth order polynomial equation to draw a normal curve which best fitted the data. The accuracy of this method was expected to be good and this was borne out by polynomial curves which closely fitted the data in most cases. A potential source of error is that non-normal nucleoid distributions occur. This would occur if a heterogeneous population of nucleoids which sediment at different rates was analysed, however this should not be a problem for analytical sucrose gradient runs where a homogeneous population of nucleoids had been selected using preparative sucrose gradients.

7.3 Results and discussion of the effect of ethidium bromide on the nucleoids from *E. coli*

To ensure that the general method for determining the superhelical density of the bacterial genome was effective, *E. coli* was used as a control. As the superhelical density of its nucleoid DNA had already been established using this method (Worcel and Burgi, 1972), a comparison of results would ensure that the experimental procedures used were effective. Nucleoids were produced, sucrose gradients run and fractions determined for their nucleic acid content as described in sections 2.13.1, 2.13.4, 2.13.5, and 2.13.6. A typical result for *E. coli* nucleoids and phage T4 particles run on sucrose gradients with different amounts of ethidium bromide is shown in figure 7.3.1.

To ensure reproducibility of sucrose gradient runs Phage T4 particles were used as an internal standard for all sucrose gradient/ethidium bromide experiments involving the *E. coli* nucleoid (figure 7.3.1). The phage's mobility in sucrose gradients was not affected by ethidium bromide and almost invariably peaked at the same sucrose gradient fraction.

Experiments to determine the superhelicity of the *E. coli* genome were conducted in triplicate. An example is shown in figure 7.3.2.

In two of the experiments the mobility of the nucleoid was maximally retarded at 2.5ug/ml (6.3uM) ethidium bromide and in the third experiment maximal retardation was at 2.0ug/ml (5.0uM) ethidium bromide. These results suggested that with increasing concentrations of ethidium bromide negative supercoiling of the nucleoid decreased until a value of

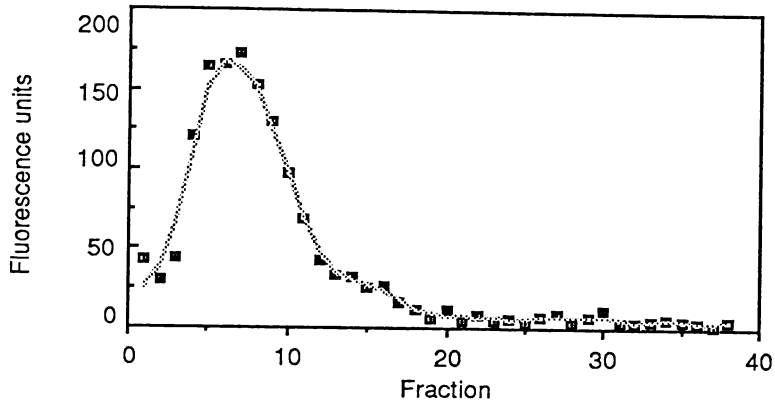


Figure 7.3.1 (a) Sedimentation profile of phage T4 analysed on a 10-30% sucrose gradient. Fraction 0 corresponds with the top of the sucrose gradient

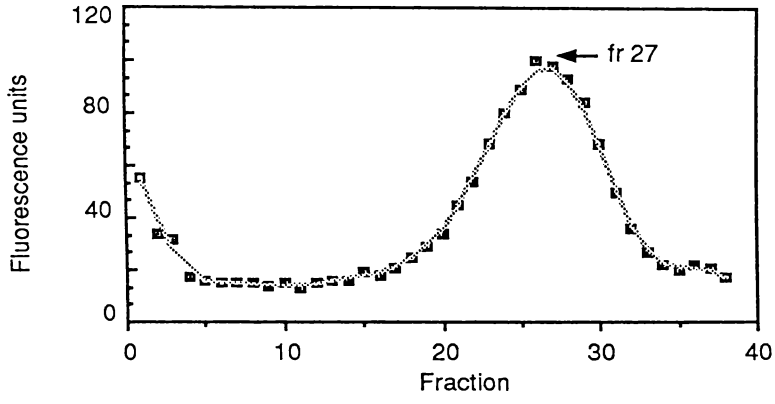


Figure 7.3.1 (b) Sedimentation profile of *E. coli* nucleoids on a 10-30% sucrose gradient. 0ug/ml ethidium bromide.

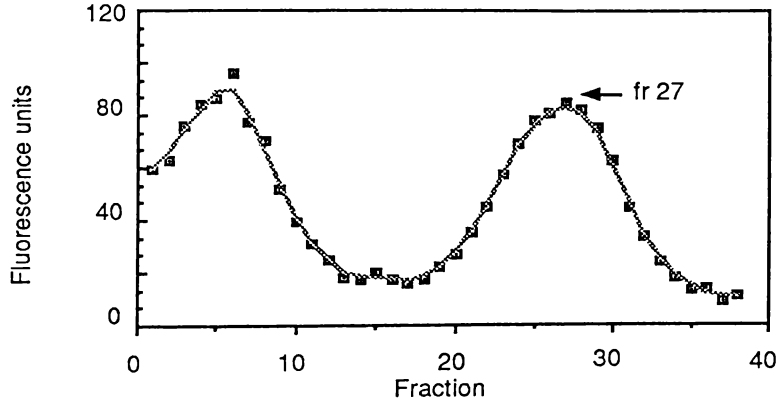


Figure 7.3.1 (c) Sedimentation profiles of phage T4 and *E. coli* nucleoids on a 10-30% sucrose gradient. 0ug/ml ethidium bromide.

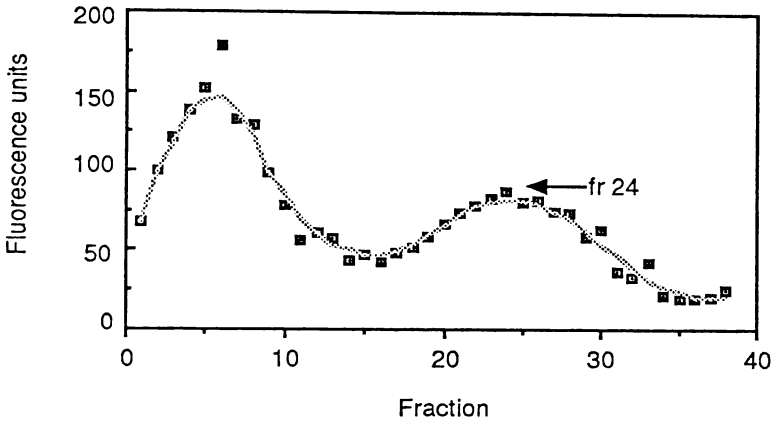


Figure 7.3.1 (d) Sedimentation profiles of phage T4 and *E. coli* nucleoids on a 10-30% sucrose gradient. 2ug/ml ethidium bromide.

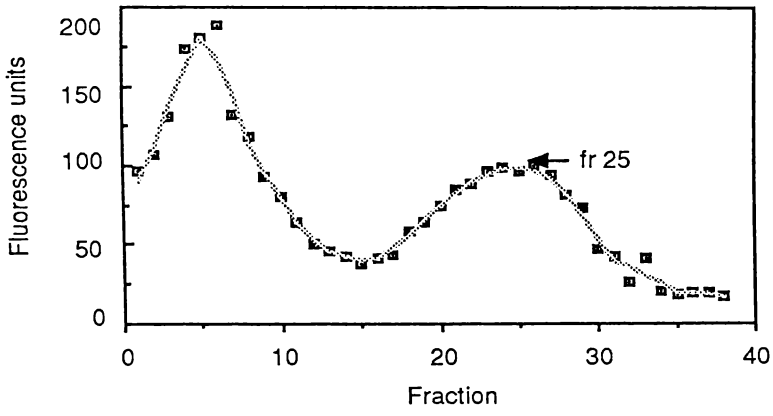


Figure 7.3.1 (e) Sedimentation profiles of phage T4 and *E. coli* nucleoids on a 10-30% sucrose gradient. 3ug/ml ethidium bromide.

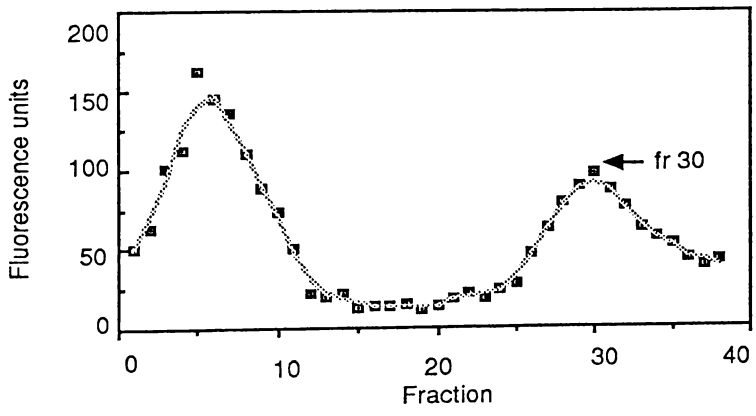


Figure 7.3.1 (f) Sedimentation profiles of phage T4 and *E. coli* nucleoids on a 10-30% sucrose gradient. 10ug/ml ethidium bromide.

approximately 2-2.5ug/ml (5.0-6.3uM) ethidium bromide was reached. At this point the nucleoid ran at its slowest through a sucrose gradient presumably due to the absence of negative or positive supercoiling. As the concentration of ethidium bromide was further increased positive supercoiling of the nucleoid DNA occured and this correlated with an increasing mobility of the nucleoid structure. Mobility of the nucleoid no longer increased at concentrations above 8 to 10ug/ml of ethidium bromide, presumably due to saturation of DNA with the drug where further supercoiling of DNA was energetically unfavourable. In all three experiments it was shown that the nucleoid was negatively supercoiled with a superhelical density of -0.18 to -0.19 (see section 2.13.6 for calculations). This correlates well with previously published results of -0.18 for the *E. coli* nucleoid (Dworsky, 1976; Pettijohn and Hecht, 1974).

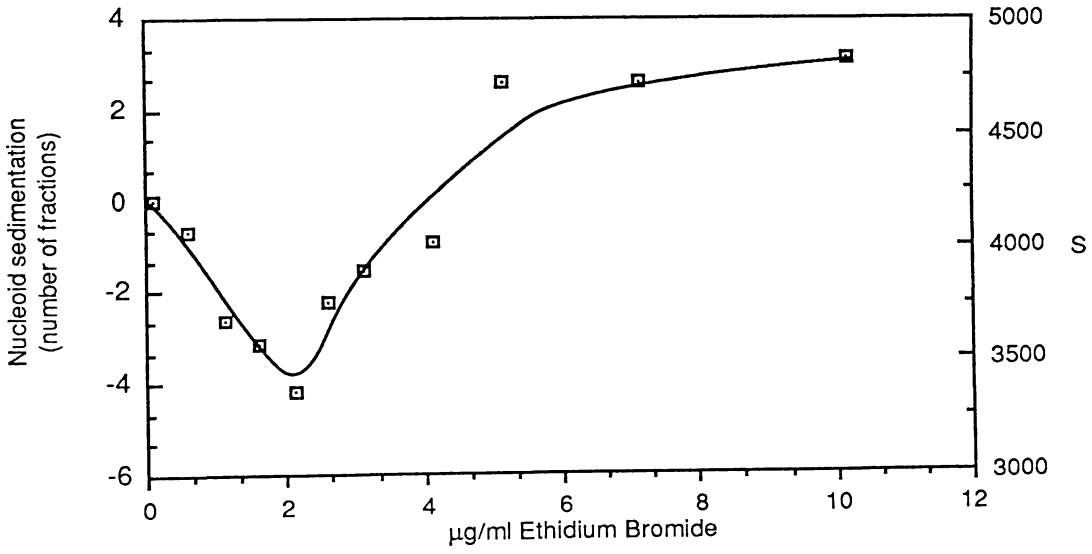


Figure 7.3.2 Ethidium bromide sedimentation profile of the *E. coli* nucleoid.

The published sedimentation rate for membrane associated nucleoids produced at low temperature (10°C) conditions is 3200S (Dworsky, 1976; Stonington and Pettijohn, 1971). The reason for the discrepancy between the published results and the result of 4100S from these experiments is unknown though one possibility is that the strain of *E. coli* is important. Published experiments were carried out with a RNase I mutant (strain D.10) whereas these results were obtained with a K12 F^- strain.

7.4 Results, and discussion of results for *Sulfolobus solfataricus*

7.4.1 Lysis of S. solfataricus

A number of different lysis protocols were tried in an attempt to isolate nucleoids from *S. solfataricus*. The main variables examined were; the growth phase of cells, lysis solutions including high salt and low salt lysis mixes, and lysis conditions including lysis temperature and time. The general procedures are described in section 2.13.2 and are based on two published procedures which have been used to isolate nucleoids. The ease with which cells lysed was related to the growth phase of the cells . Stationary phase cells (OD_{600}>0.5) lysed within 5min under both high and low salt conditions and a highly viscous solution resulted. To fulfil the criteria for production of 'nucleoids', log phase cells with an OD_{600} of 0.3-0.35 were found to give the best results. Both high salt and low salt lysis mixes were found to produce 'nucleoids'. These structures were more stable under high salt conditions than low salt conditions where the preparation became slightly viscous after 2hr at 4°C. A method used to produce nucleoids from *Bacillus licheniformis* (Sloof *et al.*, 1983) which uses neither salt, polyamines or magnesium was also attempted. This method gave similar results to the previously mentioned high and low salt procedures.

The time and temperature of lysis was varied to obtain optimum lysis without viscosity. Temperatures of 10°C, room temperature (approximately 20°C) and 60°C were used for periods of 2min to 45min. The results at 10°C and room temperature were similar and 'nucleoids' predominated after 5min of lysis. At 60°C, non viscous solutions were obtained in 2-5min and after 5min incubation increasing viscosity of the solution occurred. Consistent results were obtained in producing 'nucleoids' by using mid-log cells (OD_{600} 0.30-0.35) and lysing in high salt conditions for 5min at 10°C. These conditions were used in a series of experiments to determine if the mobility of 'nucleoids' in sucrose gradients was affected by ethidium bromide or netropsin (sections 7.4.3 and 7.4.4).

7.4.2 Sucrose gradient trials on 'nucleoids' from *Sulfolobus solfataricus*

'Nucleoids' were produced and run on ethidium bromide and netropsin sucrose gradients and the nucleic acid content of fractions determined as previously described (sections 2.13.2, 2.13.4, 2.13.5, and 2.13.6).

Sucrose gradients for these experiments were run at 9,600g for 6min. Under these conditions 'nucleoids' ran in the middle of the sucrose gradient whereas whole cells sedimented at the bottom of the tube. A brown layer consisting of cytoplasmic material remained at the top of the gradient. Reverse gyrase activity was determined for both this cytoplasmic fraction and for the 'nucleoid' fraction. Per milligram of protein (as determined by Bradfords assay) the cytoplasmic material exhibited 30x as much reverse gyrase activity as the 'nucleoid' material. Spectrophotometric traces were also conducted on these fractions. The cytoplasmic fraction peaked at 268nm and had a 280/260 ratio of 0.88 which represents about 4.9% nucleic acid (Dawson *et al.*, 1974). It is likely that the nucleic acid in this fraction is predominantly RNA species associated with cytoplasmic contents. The 'nucleoid' fraction peaked at 258nm and had a 280/260 ratio of 0.58 which represents at least 20% nucleic acid (Dawson *et al.*, 1974). It is likely that the nucleic acid in this fraction is predominantly chromosomal DNA as this fraction when heated with 1% SDS becomes extremely viscous. This is in contrast to the upper cytoplasmic fraction which showed no increase in viscosity when subjected to the same conditions.

Under the conditions of centrifugation phage T4 remained at the top of the gradient and so could not be used as a marker. Consistency of sucrose gradient runs was thereby ensured by running the 'nucleoid' on a sucrose gradient without ethidium bromide or netropsin for each run. It was assumed that inter-run comparisons could be made if the 'nucleoid' ran at the same position in each run.

It was estimated that the *S. solfataricus* 'nucleoid' was sedimenting at approximately two and a half times the speed of the *E. coli* nucleoid. This would seem to imply that the *Sulfolobus* structure was not a true nucleoid as this would be expected to sediment slower than the *E. coli* nucleoid. A lower sedimentation co-efficient was expected as the genome sizes of archaebacteria are reported to be somewhat smaller than the *E. coli* genome (Brown *et al.*, 1989). An alternative explanation for the fast sedimentation rate observed for the *Sulfolobus* 'nucleoid' is that the structure is a true nucleoid but incorporates large amounts of membrane

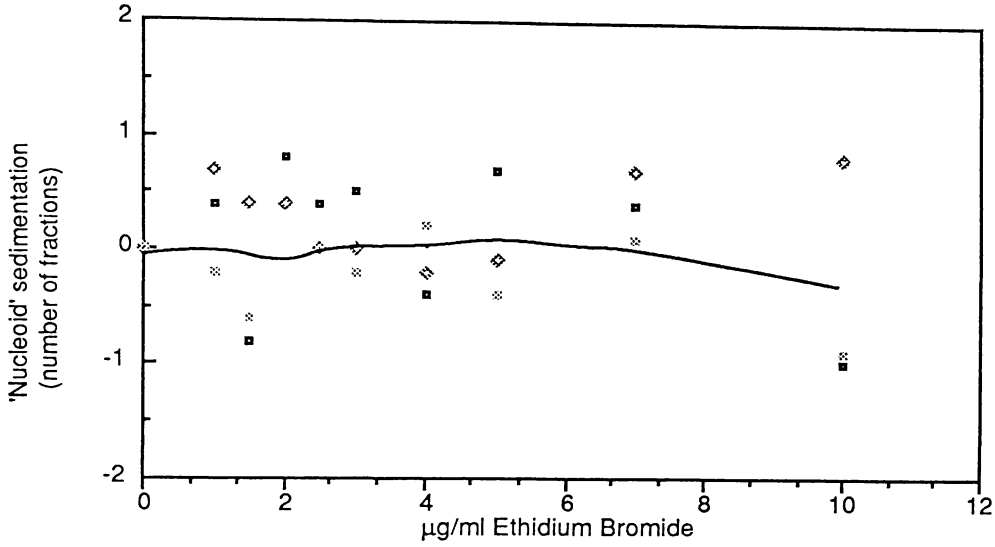


Figure 7.4.1 Ethidium bromide sedimentation profile of the *S. solfataricus* 'nucleoid'. The line delineates the smoothed means of all three sets of data.

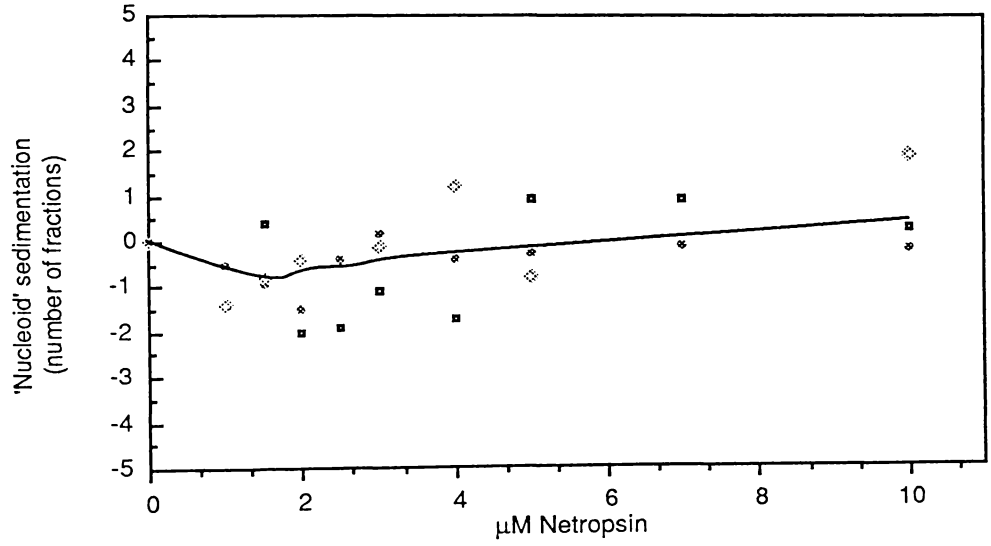


Figure 7.4.2 Netropsin sedimentation profile of the *S. solfataricus* 'nucleoid'. The line delineates the smoothed means of all three sets of data.

material. This could make a significant difference to the sedimentation co-efficient as the membrane attached nucleoid from *E. coli* sediments at about twice the speed of the non membrane attached nucleoid (Worcel and Burgi, 1974). To differentiate between the above two possibilities ethidium bromide and netropsin sucrose gradient experiments were performed.

7.4.3 The effect of ethidium bromide on the mobility of 'nucleoid's' from *S. solfataricus*

Experiments to determine whether changing concentrations of ethidium bromide affected mobility of the 'nucleoid' were conducted in triplicate. The pooled results of these experiments are shown in figure 7.4.1. A change in mobility of just over 1 fraction in a total of 40 occurred over the range of ethidium bromide concentrations used. This is within the limits of variability expected for this technique and contrasts strongly with the *E. coli* nucleoid under the same conditions where a maximal shift in mobility of 8 fractions in a total of 40 was obtained. From these experiments it was concluded that increasing concentrations of ethidium bromide had no significant effect on the mobility of the 'nucleoid' from *S. solfataricus*.

7.4.4 The effect of netropsin on the mobility of 'nucleoids' from *S. solfataricus*

Experiments to determine whether changing concentrations of netropsin affected mobility of the nucleoid type structure were conducted in triplicate. The pooled results of these experiments are shown in figure 7.4.2. No significant decrease or increase in mobility was observed. The total change in mobility between fractions was just over 1 in 40 fractions and this small change was most likely due to random fluctuations in sucrose gradient fraction collection. From this set of experiments it was concluded that netropsin, under the concentrations and conditions used, had no effect on the mobility of the 'nucleoid' from *S. solfataricus*.

7.4.5 The effect of lysis conditions on the mobility of 'nucleoids' through sucrose gradients and electron microscopy of results

Further characterization of the 'nucleoid' from *S. solfataricus* was achieved by varying the lysis conditions and running the 'nucleoids' produced on 10-30% (w/v) sucrose gradients. The resulting peak fractions were then prepared for electron microscopy (see Section 2.9.7).

'Nucleoids' produced under different temperature/time lysis conditions displayed different mobilities on sucrose gradients (figure 7.4.3). Under the conditions at which ethidium bromide and netropsin sucrose gradient runs were carried out (10°C, 5min) a single peak (peak C) was consistently observed which ran near the bottom of 10-30% (w/v) sucrose gradients under centrifugation conditions of 5,400g and 15min. When the incubation time at 10°C was lengthened to 30min a second peak (peak B) was observed which sedimented at a slower rate. This second peak can be faintly discerned from sedimentation profiles of 'nucleoids' produced at 10°C and 5min lysis conditions (figure 7.4.3). Under lysis conditions of 60°C for 5min a different pattern was noticed where a peak (peak A) sedimented near the top of the sucrose gradient. Phase contrast microscopy of peak fractions from these three lysis experiments showed 'nucleoid' type structures which could not be distinguished from one another.

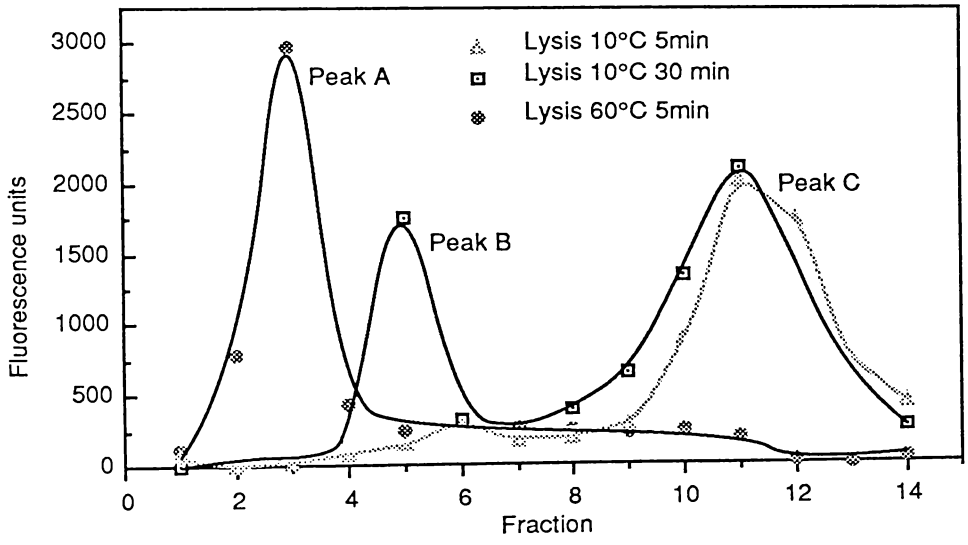


Figure 7.4.3 Sedimentation profiles of 'nucleoids' from *S. solfataricus* produced under different lysis conditions. Fraction 1 corresponds with the top of the sucrose gradient.

Electron microscopy of samples from peaks A, B, and C were conducted as described in section 2.13.7. The results are shown in figure 7.4.4. Each peak appears to correspond to a distinct structure many of which were seen in each electron microscope grid though only a single structure from each peak is pictured. A comparison of these structures can be made with the *E. coli* nucleoid which is shown in figure 7.4.5 (Freifelder, 1987).

Lysis at 60°C for 5min produced a slowly sedimenting structure (peak A) that appeared

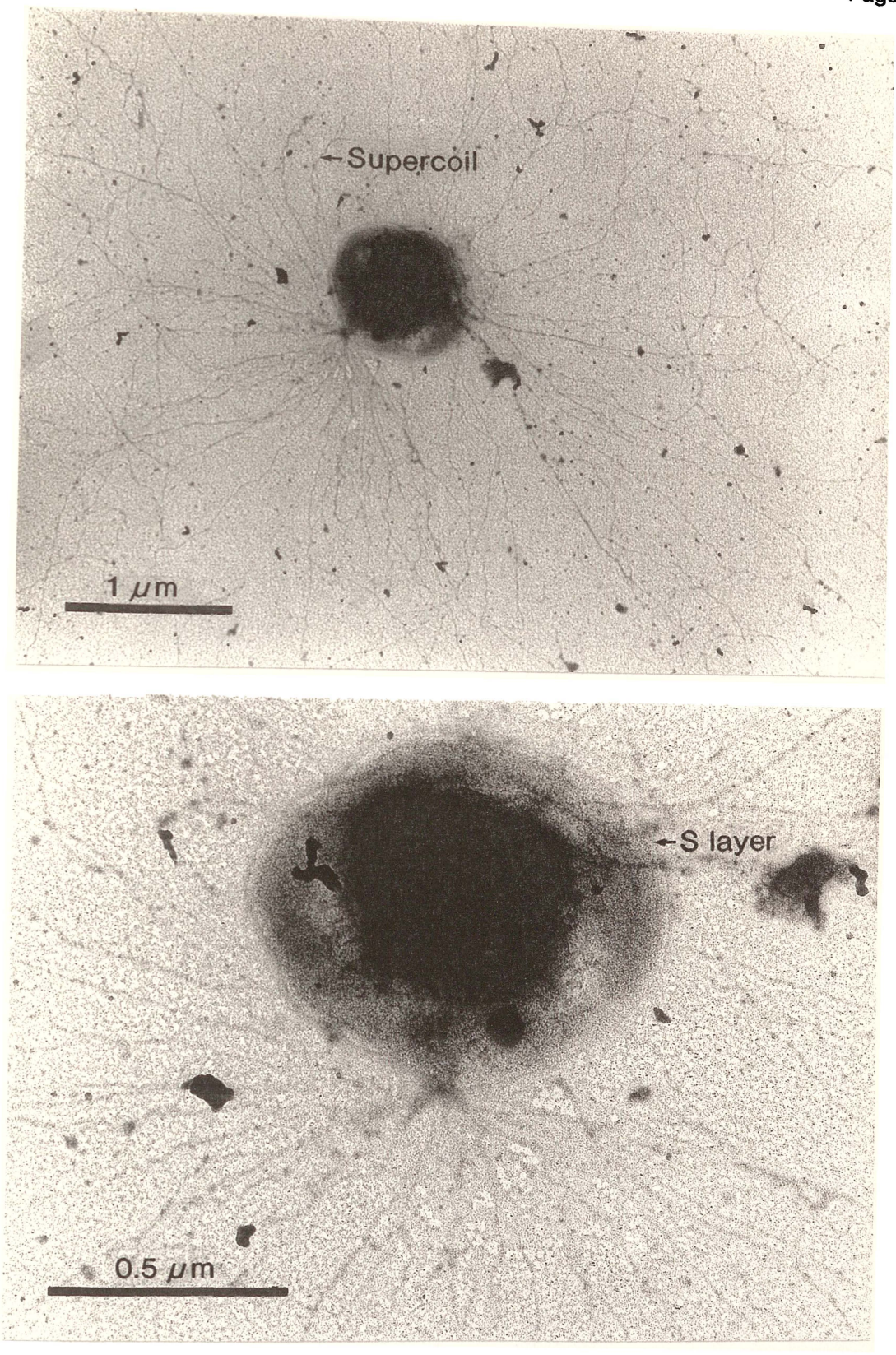


Figure 7.4.4 (a) Electron microscopy of the *S. solfataricus* 'nucleoid' from cells lysed at 60°C for 5min. Sample from peak A figure 7.4.3. The top photograph shows the total structure with DNA spreading out to 6um in diameter. The bottom photograph is a magnification showing the central structure surrounded by an S layer.

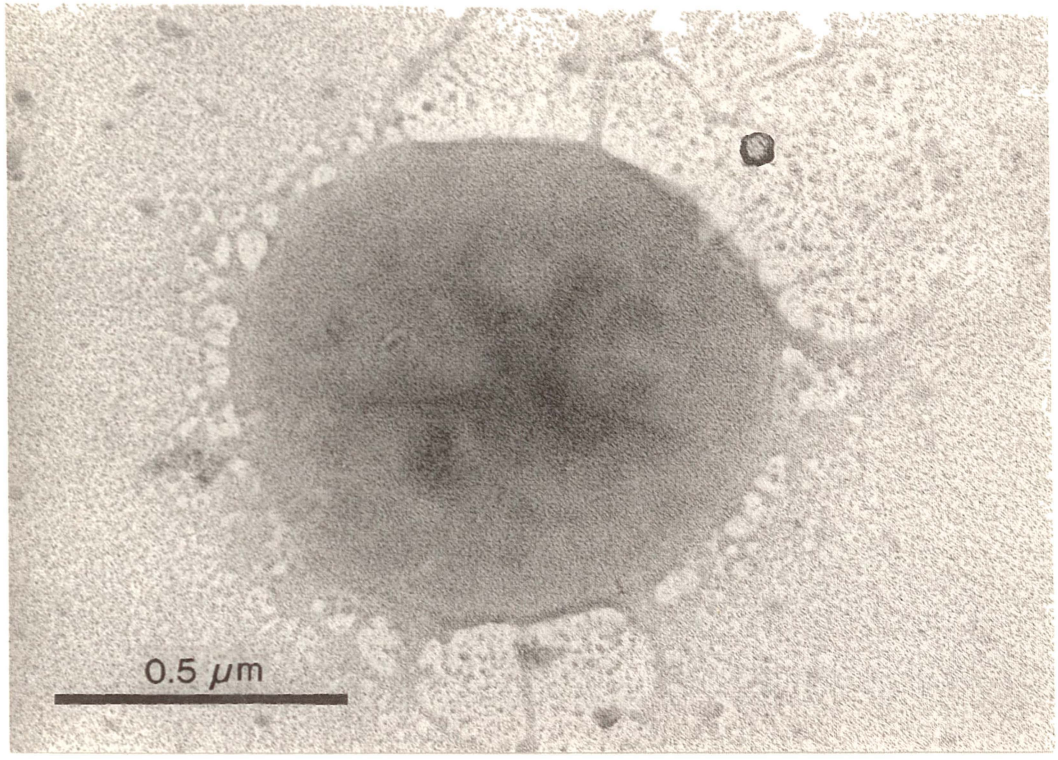


Figure 7.4.4 (b) *S. solfataricus* 'nucleoid' from cells lysed at 10°C for 30min. Sample from peak B figure 7.4.3.

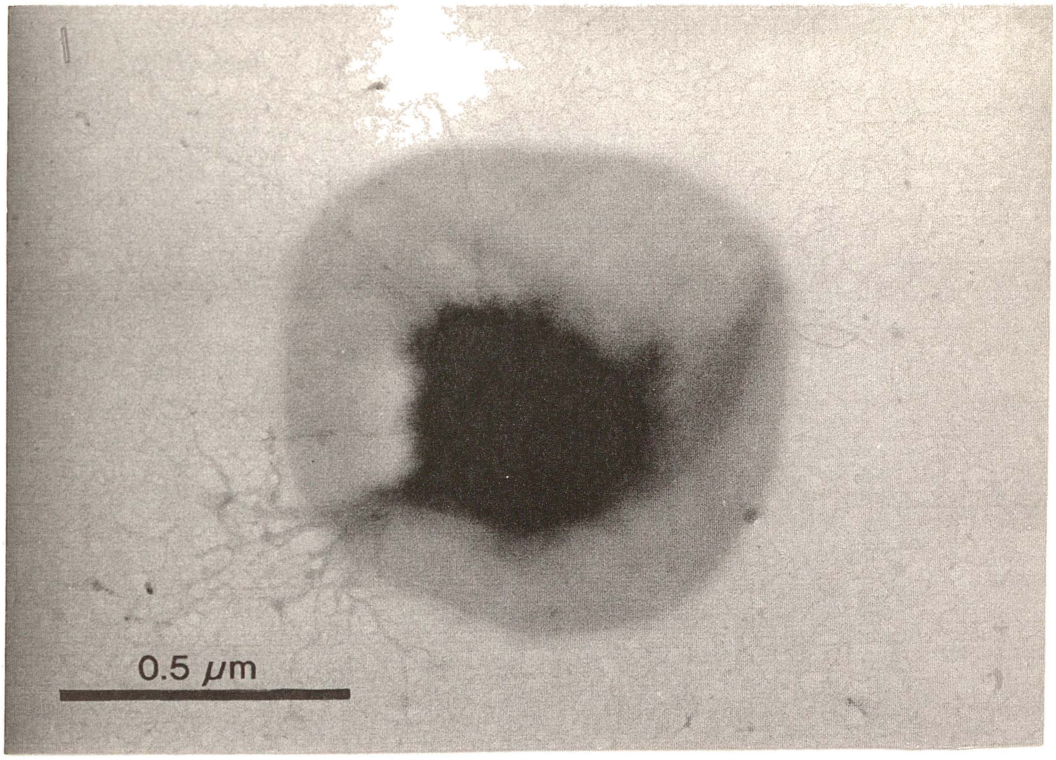


Figure 7.4.4 (c) *S. solfataricus* 'nucleoid' from cells at 10°C for 5min. Sample from peak C figure 7.4.3.

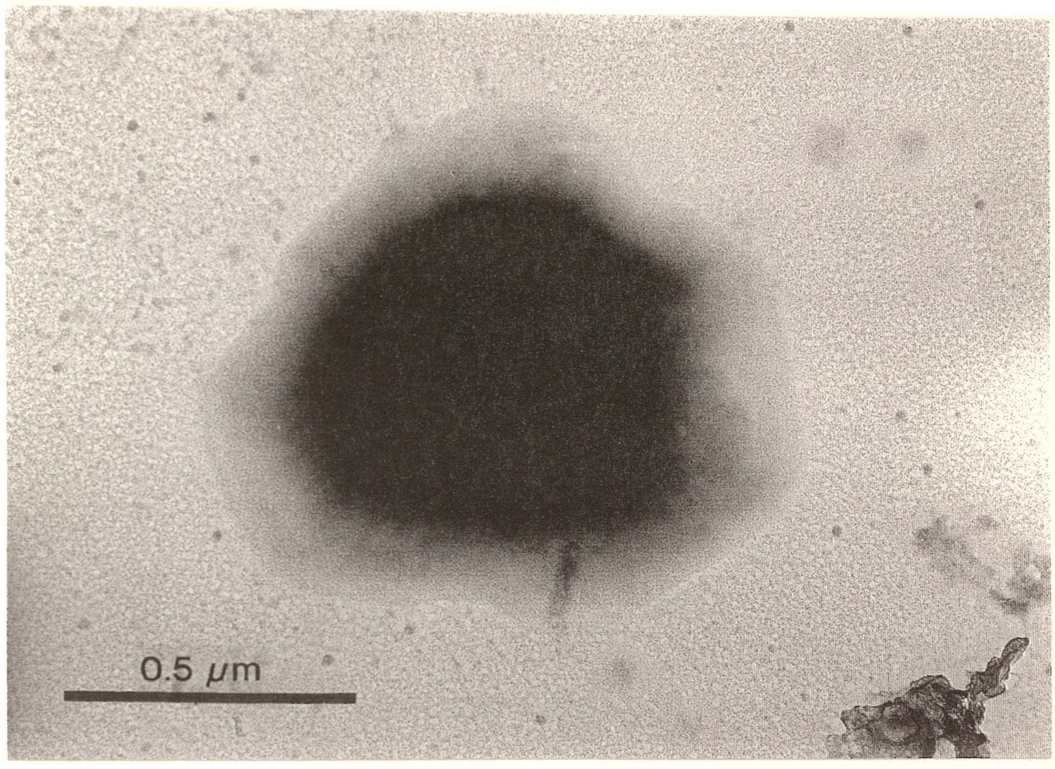


Figure 7.4.4 (d) *S. solfataricus* whole cell (under centrifugation conditions of 5400g for 15min these cells run to the bottom of a 10-30% (w/v) sucrose gradient).

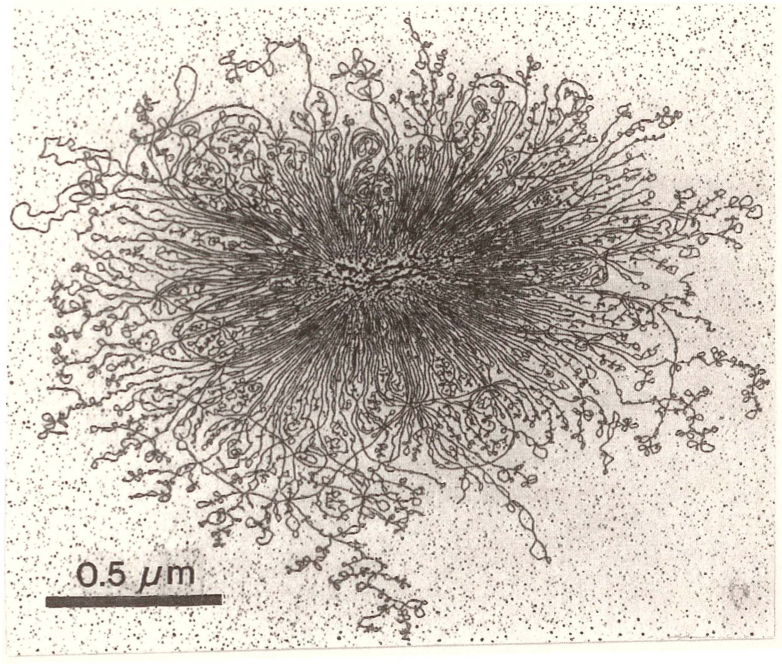


Figure 7.4.5 Electron microscopy of the *E. coli* nucleoid (reproduced from Freifelder, 1987).

under the electron microscope to comprise of an extensive network of DNA (6.0um in diameter) spreading out from the central body (1um in diameter). Some of this DNA appears to be in a supercoiled configuration but the majority of the spread DNA does not appear to be supercoiled. Whether this is the result of the lysis procedure or results from preparation for electron microscopy was not known. Most of the DNA was likely to be within the main body of the structure as this was seen to be electron dense. Under these conditions of lysis it appears that the surface layer (S layer) of protein subunits is still present. This conclusion comes from examination of the electron micrograph (figure 7.4.4(a)) where a stratum of low electron density can be seen surrounding the cell. This has a width of 20nm which corresponds to the interstice of low electron density observed between the S layer and membrane of *Sulfolobus solfataricus* (Prüschenk and Baumeister, 1987). The integrity of both membrane and S layer must be broached in several places to allow the emergence of the observed DNA.

Peak B consists of 'nucleoids' produced at 10°C for 30min. This electron light structure exhibits no sign of protruding DNA and possibly contains little DNA or cytoplasmic material judging from its electron light character. All the structures examined from peak B had a similar circular and electron light appearance.

Peak C consists of 'nucleoids' produced at 10°C for 5 and 30min. All of these structures displayed a 'poached egg' appearance and were essentially circular in contrast to the lobed forms of whole cells. It seems that the membrane/cell wall integrity of these structures was not entirely intact as evidenced by what appears to be DNA strands protruding from the structure. Similar structures are produced under low salt, 2mM spermidine conditions and thin sections of these can be seen in figure 7.4.6. These photographs clearly show the presence of an S layer surrounding the cell.

Although a precise interpretation of the above electron microscope photographs is uncertain the following explanation seems the most reasonable; the circular or lobed structures seen in all electron microscope photographs are always surrounded by cell wall (S layer) material. The subunit structure of this S layer is not clear in these photographs compared with published electron microscope photographs (Baumeister, *et al.*, 1989; Weiss, 1974). This could be the result of the staining procedure as Weiss, 1974 and Baumeister, *et al.*, 1989 used uranyl

acetate staining whereas platinum shadowing was used for these preparations. Furthermore a platinum shadowed *Sulfolobus acidocaldarius* cell, in which no subunits can be discerned, is pictured in Brock (1986). Under the conditions of lysis chosen for these experiments it seems likely that while the cell wall remains intact the cell membrane dissolves (Weiss, 1974). This allows the exit of cytoplasmic material including the electron dense ribonuclear protein particles (Weiss, 1974). This material remains at the top of the sucrose gradient as a brown layer. The resultant structure (peak C) runs slower than intact cells due to the decrease in density which results from the loss of the cytoplasmic component. At extended lysis times at moderate temperatures a proportion of these structures have S layers of insufficient integrity to contain the chromosomal DNA which previously had remained within the structure. It is proposed that the structure that results (peak B) comprises only the cell wall sacculus. This structure would be less dense than the structures from peak C and hence migrate more slowly on a sucrose gradient.

At high temperatures and short incubation times (peak A) it is proposed that the observed structures consist of a membraneless cell whose S layer has also been broached in places. This has allowed the escape of not only the cytoplasmic material but also a proportion of the chromosomal DNA which is spread around the structure and remains attached to it. This results in a slowly sedimenting structure (peak A) due to the large increase in volume brought about by the spread DNA.

Support for the above hypothesis, that it is the cell membrane that dissolves leaving the S layer largely intact, comes from a paper by Weiss (1974). This paper shows that with *S. acidocaldarius*, lysis at room temperature for 20min with a non ionic detergent (2% Triton X-100) produced cells in which the cell wall remained largely intact whereas the membrane had dissolved (figure 7.4.7). Under electron microscopy the cytoplasm was phase light compared with the untreated cell and this was taken to mean the loss of ribonuclear protein particles. It was also noted that removal of the membrane resulted in the loss of the characteristic cellular shape and this was thought to be due to a structural association between the cell wall and membrane which stabilized both of these structures thus making the cell envelope rigid. The extrapolation of these results from *S. acidocaldarius* to *S. solfataricus* however must be taken with caution since their surface proteins are not identical (Prüschenk and Baumeister, 1987) and it appears that the

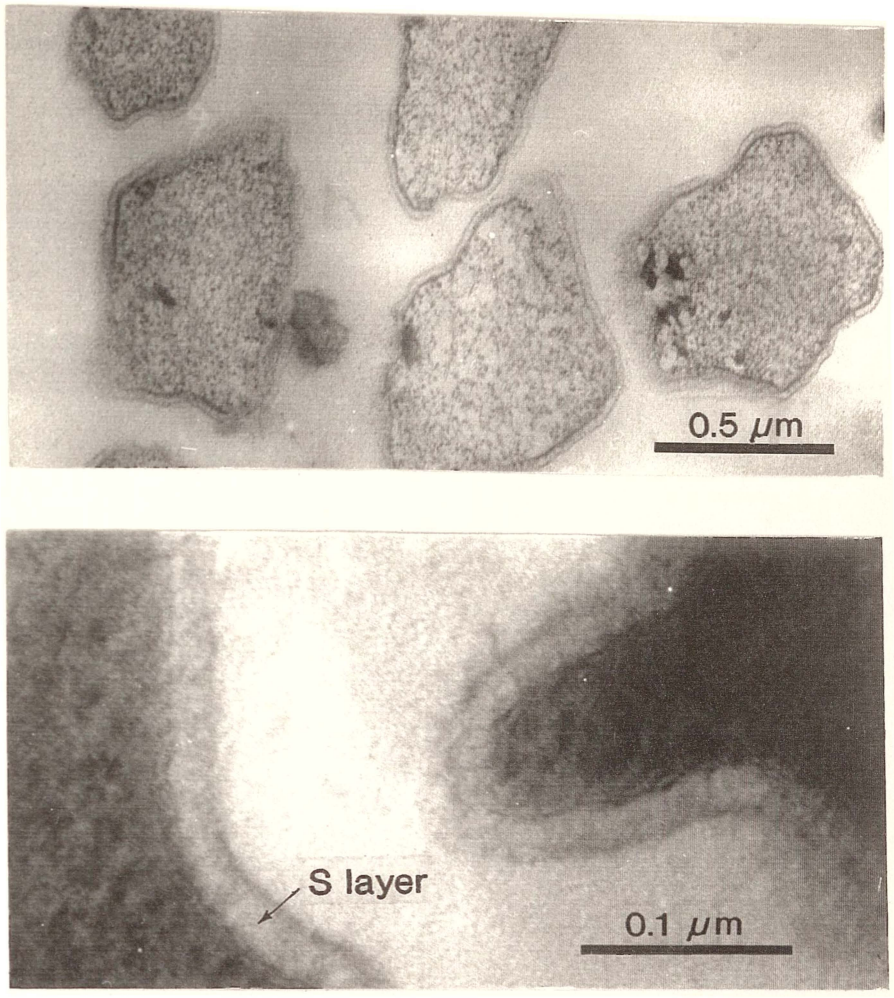


Figure 7.4.6 Thin sections of *S. solfataricus* 'nucleoids' produced under low salt 2mM spermidine conditions, lysed at 10°C (courtesy of C.Winefield).

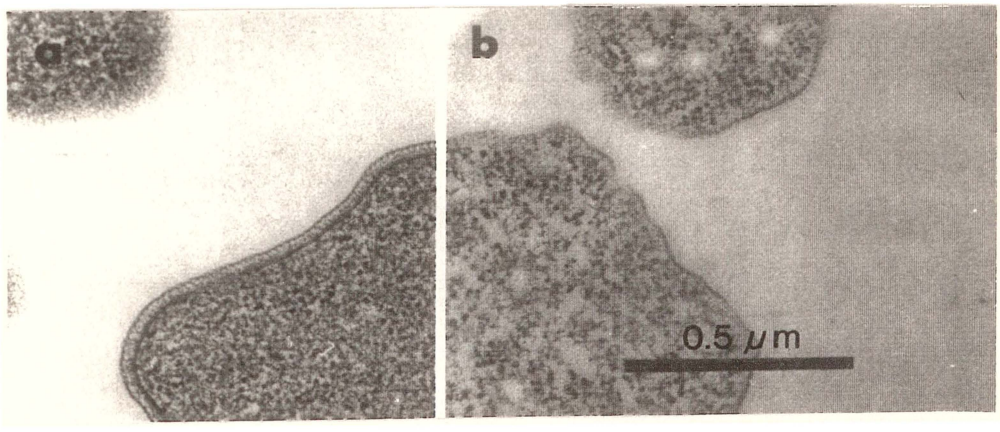


Figure 7.4.7 Thin sections of *S. acidocaldarius* . (a) Section through an intact cell; (b) section through a cell treated with 2% Triton X-100 at 23°C for 20min (reproduced from J.Bacteriology, Weiss, 1974).

S layer of *S. solfataricus* is somewhat more labile than the S layer of *S. acidocaldarius* (König and Stetter, 1986).

7.5 Results, and discussion of results for AN1

7.5.1 Lysis of AN1

AN1 cells were grown, harvested and lysed as described in sections 2.5 and 2.13.2. Lysis of cells at room temperature for 15min under both high and low salt conditions produced a clearing of the solution with no increase in viscosity. Under phase contrast microscopy the structures produced were similar in appearance to the 'nucleoid' structures of *S. solfataricus*. Lysis of cells at 60°C for 3min under low salt, 2mM spermidine conditions produced a non-viscous solution whereas under conditions employing 1M NaCl a viscous solution resulted. All subsequent experiments both at high temperatures and at room temperature were therefore carried out using the 2mM spermidine, 1% Nonidet, lysis protocol.

7.5.2 Sedimentation profiles and ethidium bromide and netropsin sucrose gradients on AN1 'nucleoids'

'Nucleoids' from AN1 produced at either 60°C for 3min or 20°C for 10min, were run on 10-30% (w/v) sucrose gradients under centrifugation conditions of 17,000g for 10min (figure 7.5.1). A similar sedimentation profile was obtained to that of *S. solfataricus* nucleoids (figure 7.4.3) in that lysis at higher temperatures produced slower sedimenting structures.

A brown layer, cytoplasmic material, was always noticed at the top of sucrose gradients and this is in accord with what was observed with *S. solfataricus*. It can be seen that at a lysis temperature of 60°C the main peak runs slower than 'nucleoids' produced at room temperature and that a smaller peak sediments at the same rate as the peak obtained from room temperature lysed material. The major difference between AN1 'nucleoid' sedimentation profiles and *S. solfataricus* 'nucleoid' sedimentation profiles is that sedimentation of AN1 'nucleoids' is much slower and is in fact comparable to the sedimentation rate of the *E. coli* nucleoid.

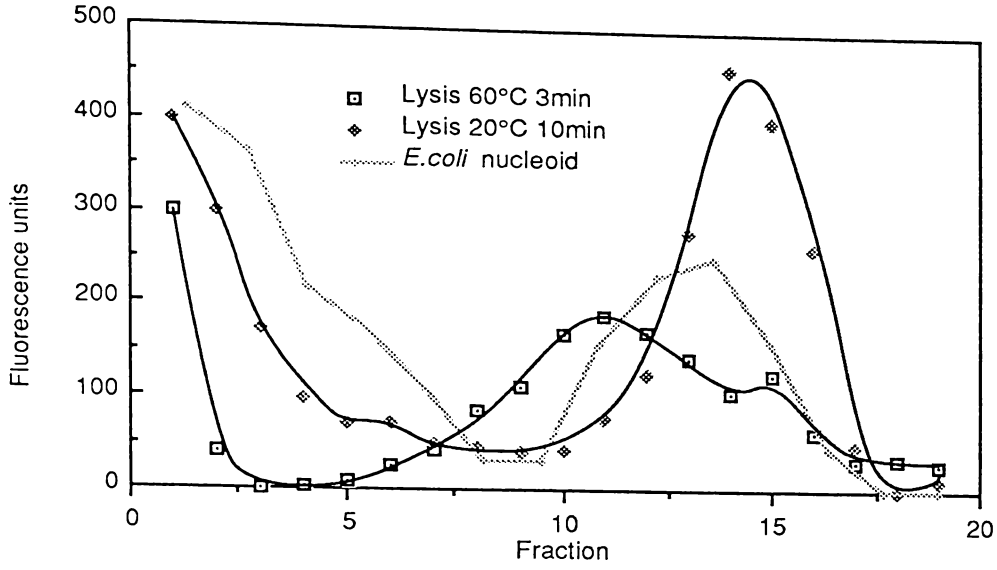
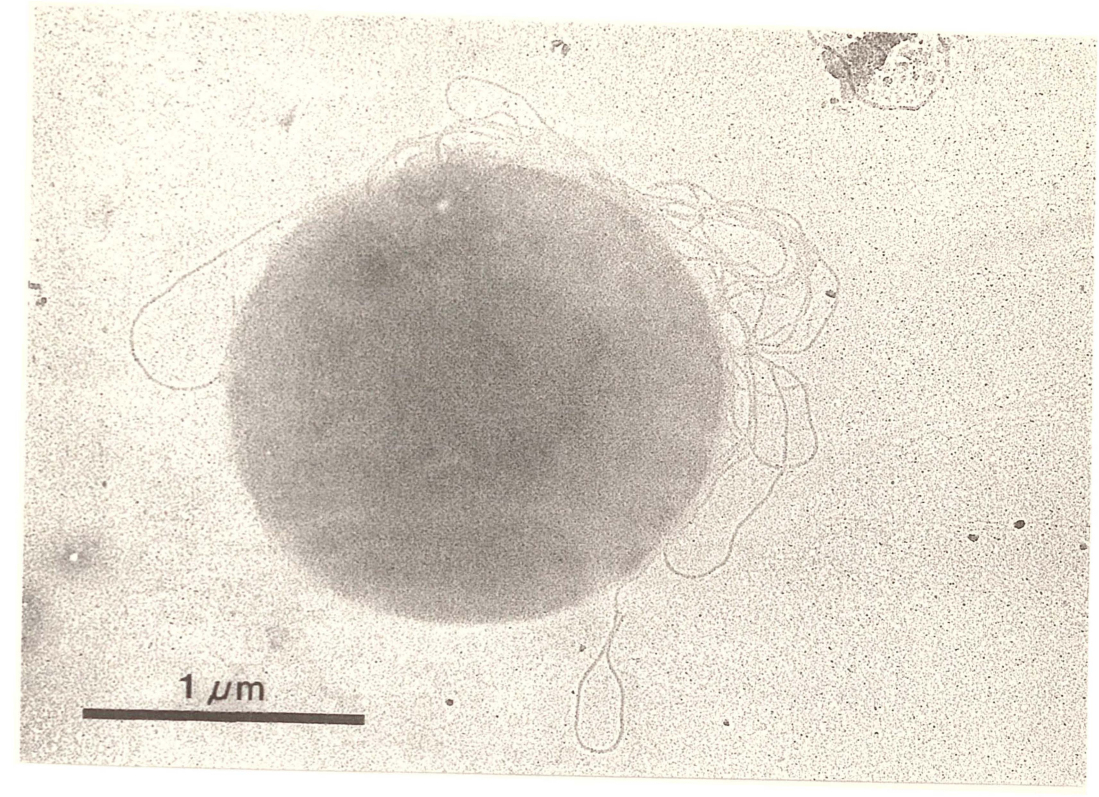


Figure 7.5.1 Sedimentation profiles of 'nucleoids' from AN1 produced under different lysis conditions. Fraction 1 represents the top of the sucrose gradient.

To further characterize the AN1 'nucleoids', electron microscopy, and ethidium bromide and netropsin sucrose gradients were used.

AN1 'nucleoids' produced at room temperature for 15min showed no signs of changes in mobility when subjected to different concentrations of ethidium bromide or netropsin on sucrose gradients. Changes in mobility did not vary by more than 2 fractions in individual experiments and no consistent patterns of mobility change versus drug concentration were noticed between experiments. Electron microscope grids were prepared from samples off the peak fraction of sucrose gradients of AN1 'nucleoids' lysed at room temperature for 15min. These grids were prepared as described in section 2.13.7 and the results are illustrated in figure 7.5.2. Many of these structures were seen per grid and no alternative structures were seen. The electron light appearance of these structures would suggest that cytoplasmic material and chromosomal DNA had been released from the structure resulting in a structure consisting primarily of cell wall material. This would be similar to the structure found in *S. solfataricus* peak B figure 7.4.3. A distinctive difference between the *S. solfataricus* and AN1 structures is the polar tuft of flagella seen on the AN1 structure. This has been previously observed in AN1 cells (H.W. Morgan personal communication). These flagella appear to be attached to the S layer and are not removed under the lysis conditions used. It seems possible that the relative slowness of sedimentation of the AN1 'nucleoid' compared with *S. solfataricus* 'nucleoids' is due to these



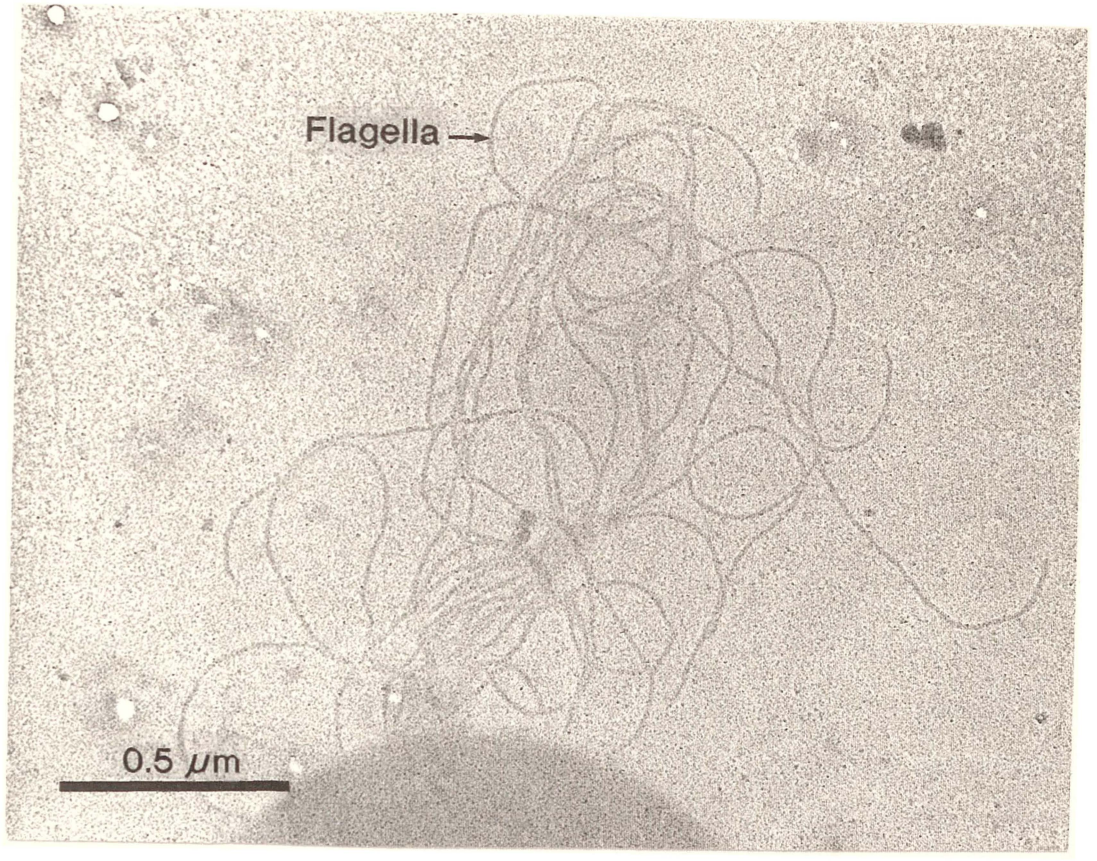


Figure 7.5.2 AN1 'nucleoid' from cells treated with 1% nonidet, 2mM spermidine at 20°C for 15min.

flagella, bearing in mind that other possible contributory factors such as size, shape and cell wall density are similar for both archaebacteria.

AN1 'nucleoids' produced at 60°C for 3min were subjected to sucrose gradients containing different levels of netropsin. Phage T4 was used as an internal marker. Mobility of the 'nucleoid' did not change by more than 2 fractions which is within the experimental variation of this technique. No pattern of increasing or decreasing mobility was noticed and it was concluded that under these experimental conditions, netropsin concentrations did not have a noticeable effect on the mobility of AN1 'nucleoids'. Electron microscopy was not performed on samples which were lysed at 60°C for 3min. However in the absence of any mobility shift in different netropsin concentrations and the similarity of results with *S. solfataricus* under room temperature lysis conditions it was assumed that high temperature lysis conditions would give similar results to that seen for *S. solfataricus* peak A figure 7.4.3.

7.6 Discussion

Attempts to isolate nucleoids from AN1 and *S. solfataricus* were unsuccessful as was demonstrated by; the lack of true nucleoid structures when visualised by electron microscopy, and the ineffectiveness of ethidium bromide and netropsin in altering the mobility of the nucleoid type structures.

The failure to isolate nucleoids is disappointing in that the possibility of elucidating the supercoiled topology of the genome did not occur. The discovery of a positively supercoiled genome would be interesting not only for the uniquely supercoiled properties of such a genome but also for the questions it would pose regarding gene regulation in such a genome and the unique phylogenetic status of these organisms. It is known that the state of negative supercoiling has profound effects on gene regulation and transcription (Drlica, 1984; Sternglanz *et al.*, 1981). This is in part due to dependency on the state of supercoiling for binding of RNA polymerases to promoters and binding of gene regulatory proteins to DNA (Pruss and Drlica, 1989). If sulphur metabolizing archaebacteria have evolved with a positively supercoiled genome they may well be expected to contain unique gene regulatory proteins and systems.

Reddy and Suryanarayana, 1988 report the isolation of nucleoids from *S. acidocaldarius*

using lysis conditions identical to that used for low salt lysis of *S. solfataricus* in this study. It is proposed here that the structures they obtained were in fact similar to the structures obtained at low temperatures for *S. solfataricus* in this study. This is suggested by the protein-DNA ratio for the nucleoid (*sic*) of *S. acidocaldarius* (protein:DNA, 7:1, (Reddy and Suryanarayana, 1988)) which is high in comparison to the membrane bound *E. coli* nucleoid (protein:DNA, 1:1, (Pettijohn *et al.*, 1973))

The failure to obtain nucleoids in this study may be attributed to failure of the cell wall to disintegrate under the lysis conditions employed and perhaps to membrane dissolution which may adversely affect the integrity of membrane attached nucleoids. Further research could bring about success in the isolation of nucleoids from sulphur metabolizing archaebacteria bearing in mind that their cell wall and membrane are markedly different from *E. coli* and methods used to isolate *E. coli* nucleoids are unlikely to work without some degree of modification. The critical difference between methods used for eubacteria and those that can be used for archaebacteria is the use of lysozyme which can specifically digest the cell wall of eubacteria without detrimental effects to the cell membrane and the nucleoid. A method is needed to remove the S layer before membrane dissolution is attempted in archaebacteria. As the S layer consists of protein subunits, protein denaturents may work and indeed 6M guanidine HCl has been used to remove this layer from *S. acidocaldarius* (Weiss, 1974). This is likely however to have detrimental effects on protein-DNA interactions and hence nucleoid stability. It is possible that time limited proteolysis with a protease may be sufficient to remove S layer material without detrimental effects on the nucleoid. Procedures could then be developed for the gentle dissolution of the membrane perhaps by using non-ionic detergents. This may be sufficient to release a nucleoid structure.

CHAPTER EIGHT

CONCLUSION

This thesis was initiated to examine some aspects of thermophilic bacteria which contribute towards the stability of their DNA at high temperatures. Factors which are known to increase DNA thermostability include guanine plus cytosine content, DNA binding proteins, an increase in ionic concentration, and, for topologically restrained DNA, the supercoiled state of such DNA. Many thermophilic archaebacteria possess a guanine plus cytosine content which is low in comparison to many mesophilic bacteria e.g. *Sulfolobus* G+C ~37%, and *Pyrococcus* G+C ~38%. This observation rules out the possibility that thermophilic archaebacteria contain enchanced levels of guanine and cytosine as a general mechanism against thermal denaturation. However most, if not all, thermophilic archaebacteria contain significant quantities of polycations (Friedman and Oshima, 1989) whose contribution to the ionic conditions within the cell is expected to increase the thermostability of DNA. The presence of DNA binding proteins, which prevent the denaturation of DNA at high temperatures has also been demonstrated in a number of thermophilic archaebacteria (Reddy and Suryararayana, 1988; Searcy, 1986) and such proteins are likely to significantly contribute towards the observed thermostability of DNA in these archaebacteria. The positive (or reduced negative) supercoiling of topologically constrained DNA is another factor which could be involved in enhancing the thermostability of DNA from thermophilic archaebacteria by the tightening of the DNA helix. Reverse gyrase is the only known topoisomerase which can actively tighten (or positively supercoil) DNA and it was this enzyme, and its postulated effects on the genome of sulphur metabolizing archaebacteria, which was examined in detail in this thesis.

The purification and characterization of reverse gyrase from two taxonomically distinct sulphur metabolizing archaebacteria (*Sulfolobus solfataricus* and *Thermococcus* isolate AN1) showed that no significant difference could be discerned between them or between the reverse

gyrase enzyme isolated from *S. acidocaldarius* (Nakasu and Kikuchi, 1985) or *Desulfurococcus amylolyticus* (Slesarev, 1988). Reverse gyrase from all of these organisms has optimum activity at a temperature of 75-90°C, a pH of ~6.5 (75°C), ionic conditions of 150-250mM NaCl or KCl and ~10mM $MgCl_2$, and with an energy requirement of > 10uM ATP. They are high molecular weight (120-135kDa) monomeric enzymes capable of positively supercoiling cccDNA and with a preference for ssDNA. The reverse gyrase enzyme was found to be similar to the eubacterial type I topoisomerases in many respects including substrate and cleavage site specificity (Kovalsky *et al.*, 1990; Slesarev and Kozyavkin, 1990) while it appears to exhibit little similarity to the eukaryotic type I topoisomerases.

Reverse gyrase has been found in all sulphur metabolizing archaebacteria studied but was not found in other bacterial groups (Collin *et al.*, 1988), however this does not preclude the possibility that reverse gyrase is present in other, especially archaebacterial, organisms. The presence of reverse gyrase in the two major branches of the archaebacterial kingdom (if Achenbach-Richter *et al.*, 1988 are correct in placing the *Thermococcales* with the methanogen/halophile branch) demonstrate that this enzyme is a feature of the primeval archaebacterium and as such is one of the most ancient enzymes studied. It thus seems quite plausible that other type I topoisomerases, especially eubacterial type I topoisomerases, may have derived from reverse gyrase.

Attempts to determine the *in vivo* effect of reverse gyrase on the supercoiled state of genomic DNA did not succeed, which was unfortunate as such information would have been of value not only to clarify the role of reverse gyrase but also to determine any correlation between DNA supercoiling and genome thermostability. Despite the lack of any direct evidence, the activity of reverse gyrase *in vitro* and the presence of positively supercoiled viral DNA within *S. acidocaldarius* (Nadal *et al.*, 1986), suggests that reverse gyrase is active *in vivo* to positively supercoil genomic DNA. This suggests a role for reverse gyrase which is to control DNA duplex stability near its minimum level in conjunction with a relaxing topoisomerase (Slesarev and Kozyavkin, 1989). Under conditions of high temperature the DNA helix will be destabilized to a greater extent and so require an increase in linking number (catalysed by reverse gyrase) to combat this effect.

The possibilities for future research in the area of topoisomerases and their associated effects on DNA supercoiling in thermophilic archaebacteria include:

Further research into determining the *in vivo* state of supercoiling in these organisms using such methodologies as the binding of trimethylpsoralen to DNA or determining the effect of positive supercoiling on the rate of transcription of selected genes *in vitro* and using this to calibrate the superhelical density of DNA *in vivo* by determining the *in vivo* rate of gene transcription. Such methodologies have worked well in organisms containing negatively supercoiled DNA (Sinden *et al.*, 1980; Borowiec and Gralla, 1987) but could require modification to work on thermophilic organisms possessing potentially positively supercoiled genomes.

A search for proteins which specifically bind to positively supercoiled DNA would be of interest and perhaps reveal how important the degree of DNA supercoiling is in the genome. Characterization of such proteins could possibly provide valuable information on unique promotor sequences or regulatory elements amongst these bacteria.

Further research to elucidate the mode of action of reverse gyrase would also be of interest and could provide information on structural movement within the enzyme catalysed by ATP hydrolysis. Such information could also clarify the relationship of reverse gyrase to other topoisomerases. Cloning and sequencing the reverse gyrase gene would also provide the information necessary to determine the relatedness of reverse gyrase to other topoisomerases.

Another potential research project is the screening and isolation of temperature sensitive reverse gyrase mutants. Such a mutant could yield a great deal of information about the role of reverse gyrase, however a methodology for the screening of reverse gyrase mutants would have to be developed before such a project could proceed.

More speculative research could be carried out to determine the effect of positive superhelicity (assuming that sulphur metabolizing archaebacteria do have positively supercoiled genomes) on gene transfer between organisms. One could postulate that the possession of positively supercoiled genomes would enhance the genetic isolation of these organisms and inhibit DNA transfer from the viruses and plasmids of negatively supercoiled organisms.

APPENDIX I

LB medium

	per litre
bacto-tryptone	10g
bacto-yeast extract	5g
NaCl	10g

The pH was adjusted to 7.0 with 5N NaOH (~0.2ml). Growth of bacteria with phage P4 was on modified Luria Broth which contained in addition to the above ingredients; 1.6ml/l $MgCl_2$ (1M), 0.5ml/l $CaCl_2$ (1M), and 10ml/l dextrose (10%), all added after autoclaving of the basic medium.

M9CA medium

	per litre
Na_2HPO_4	5g
KH_2PO_4	3g
NaCl	0.5g
NH_4Cl	1g
Casamino acids	2g

The pH was adjusted to 7.4 and sterilised by autoclaving after which; 2ml/l $MgSO_4$ (1M), 4ml/l glucose (50%), and 1ml/l $CaCl_2$, was added.

2xYT medium

	per litre
bacto-tryptone	16g
bacto-yeast extract	10g
NaCl	5g

The pH was adjusted to 7.0 with 5N NaOH and sterilised by autoclaving.

TB broth (terrific broth)

	per 900ml
bacto-tryptone	12g
bacto-yeast extract	24g
glycerol	4ml

This was sterilised by autoclaving, allowed to cool, and 100ml of 0.17M KH_2PO_4 and 0.72M K_2HPO_4 added. This solution was made by dissolving 2.31g of KH_2PO_4 and 12.54g of K_2HPO_4 in milliQ water to give a total volume of 100ml then autoclaved.

T4 phage broth

	per litre
Difco bacto-peptone	15g
Oxoid tryptone broth	8g
NaCl	8g
glucose	1g

The pH was adjusted to 7.2 with NaOH before autoclaving.

T phage nutrient agar

	per litre
Difco bacto-agar	10g
Oxoid tryptone broth	10g
NaCl	8g
glucose	1g

The pH was adjusted to 7.2 with NaOH before autoclaving. Soft T phage nutrient agar, used for overlaying, contained the same ingredients except that 6g/l of Difco bacto-agar was used.

Thermoproteus and *Desulfurococcus* medium

	per litre
yeast extract	0.3g
trypticase peptone	2.0g
$(NH_4)_2\ SO_4$	1.3g
$CaCl_2\ 2H_2O$	0.074g
$MgSO_4.7H_20$	0.28g
KH_2PO_4	0.28g
Nitsch's trace elements (Brock *et al.*, 1972)	1ml
L-Cystine	0.5g
$Na_2S.9H_20$	0.1g
resazurin (0.1%)	1.0ml

The pH was adjusted to 6.0 with 1M H_2SO_4 at 25°C and the medium prepared anaerobically by adding the L-Cystine after autoclaving and flushing with oxygen free nitrogen.

AN1 medium

	per litre
trypticase peptone	8.0g
K_2HPO_4	1.5g
$MgCl_2.6H_2O$	0.3g
NaCl	2.5g
Zeikus trace elements (Zeikus *et al.*, 1979)	5.0ml
Wolins vitamins (Wolins *et al.*, 1963)	1.0ml
Sulphur	5.0g
NaThioglycolate	0.5g
Resazurin (0.1%)	1.0ml

The pH was adjusted to 7.3 with 1M H_2SO_4 at 25°C and the medium prepared anaerobically by flushing the vessel with oxygen free nitrogen after autoclaving.

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