Aspects of the Initiation of DNA Replication in Gram-negative bacteria.

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Abstract

An 8.2kb EcoR1 fragment of E.coli DNA which suppresses mutations in the dnaA gene has been cloned. Genetic mapping, complementation and comparison with published restriction data show that this fragment carries the heatshock genes groEL and groES. Suppression appears to be the result of amplification of genes on the cloned fragment, and in some strains this is accompanied by slow growth rate, reduced resistance to nalidixic acid and cold sensitivity.

Hybridisation of E.coli oriC DNA to genomic DNA from the Enteric bacteria Enterobacter aerogenes, Enterobacter cloacae, Citrobacter freundii, Klebsiella pneumoniae, Serratia marcescens, Providencia alcalifaciens, Providencia stuartii, Proteus vulgaris, Morganella morgani and Hafnia alvei showed that:

1) oriC itself is less highly conserved than neighbouring sequences, probably those of the atp operon.

2) In Providencia alcalifaciens, Proteus vulgaris and Enterobacter aerogenes, multiple copies of DNA homologous to oriC are observed.

Attempts to clone origins of replication from E.coli, Alcaligenes faecalis, Morganella morgani, Citrobacter freundii and Vibrio natriegens are described, together with the properties of some multiply antibiotic-resistant mutants obtained during these experiments.

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Declaration

This thesis was composed entirely by myself, and the work described in it was carried out unaided, except for the following experiments: much of the gel electrophoresis which provided the data for restriction mapping of the *sidA* locus was done by Ian Oliver, and the curing curves in chapter 5 were done by Christine Henry, who also assisted in the recloning of the *sidA* fragment, and in producing some of the Southern transfers used in Chapter 4. I am indebted to both for their help.

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CHAPTER 1: THE INITIATION OF CHROMOSOMAL REPLICATION

DNA replication is considered to consist of three processes: Initiation, where the various components of the replication apparatus are assembled at the origin of replication and dispatched on their journey round the chromosome; elongation, where the assembled "replisomes" (Kornberg,1978) move (usually) in both directions round the chromosome synthesising new strands of DNA; and termination, a rather obscure process where replication forks converge, daughter chromosomes are topologically separated, and the replication apparatus disassembles.

Under normal conditions, DNA replication in *E.coli* is bidirectional and initiates at the unique origin, *oriC*, located at 84' on the *E.coli* genetic map (Masters & Broda,1971; Bird *et al*,1972; von Meyenburg *et al*,1977). Termination occurs at *terC*, diametrically opposite *oriC* (Bouche *et al*.,1982).

Initiation is the key control step in cellular DNA synthesis (Helmstetter *et al*.,1968). If *E.coli* is to survive, each newly divided cell must receive a copy of the *E.coli* chromosome, and if this is to be possible, chromosome replication must be complete by the time cell division occurs. The rate of division varies depending on the growth rate and so the rate of completion of chromosomes must be varied accordingly. Since the rate of movement of a replication fork is constant, except when cells are growing very slowly (Cooper & Helmstetter,1968), the rate of chromosome replication cannot be varied except by altering the frequency of initiation.

Initiation, elongation, termination and cell division define the C and D periods of the cell cycle (Helmstetter *et al*.,1968). C is the time taken to replicate the chromosome, the period between initiation and termination; D is the time between termination and cell division.

Donachie (1968) showed that cells achieved coordination of initiation and cell growth by initiating replication only when they reached a fixed cell mass or volume, called initiation mass, or a binary multiple thereof. After initiation is triggered, elongation proceeds for a fixed 40 minutes (at 37°C) (Cooper & Helmstetter,1968) after which protein synthesis at termination triggers events which lead to cell division 20 minutes later (Jones & Donachie,1973). During this period, the cells, if growing rapidly, may double in size and initiation will occur again. If this occurs before termination, the chromosomes will contain nested replication forks and daughter cells will receive replicating chromosomes. Consequently, at high growth rates, cells have a high ratio of origin to terminus markers, a property which allowed localisation of the origin of replication (Masters and Broda,1971; Bird et al,1972)

How this control of initiation is achieved remains one of the major unsolved problems in the biology of E.coli. Models based on the dilution of a periodically synthesised repressor (Pritchard et al.,1969), or the accumulation of an initiator (Helmstetter et al.,1968) are both tenable.

Whatever may set initiation in train, we know that its ultimate result is the formation of two replication forks. The apparatus required for DNA replication at the replication fork is known as the replisome.

The Replisome

For a replication fork to progress a number of biochemical activities must be coordinated there. Kornberg (1978,1982) suggests that these are gathered together in a moving structure called a replisome. The synthetic activities are polymerisation of the leading strand in an overall 3' - 5' direction and polymerisation of the lagging strand in an overall 5' - 3' direction, the latter requiring intermittent synthesis of an RNA primer by DNA primase followed by extension of this primer by DNA polymerase III to overcome the problem that DNA polymerase cannot

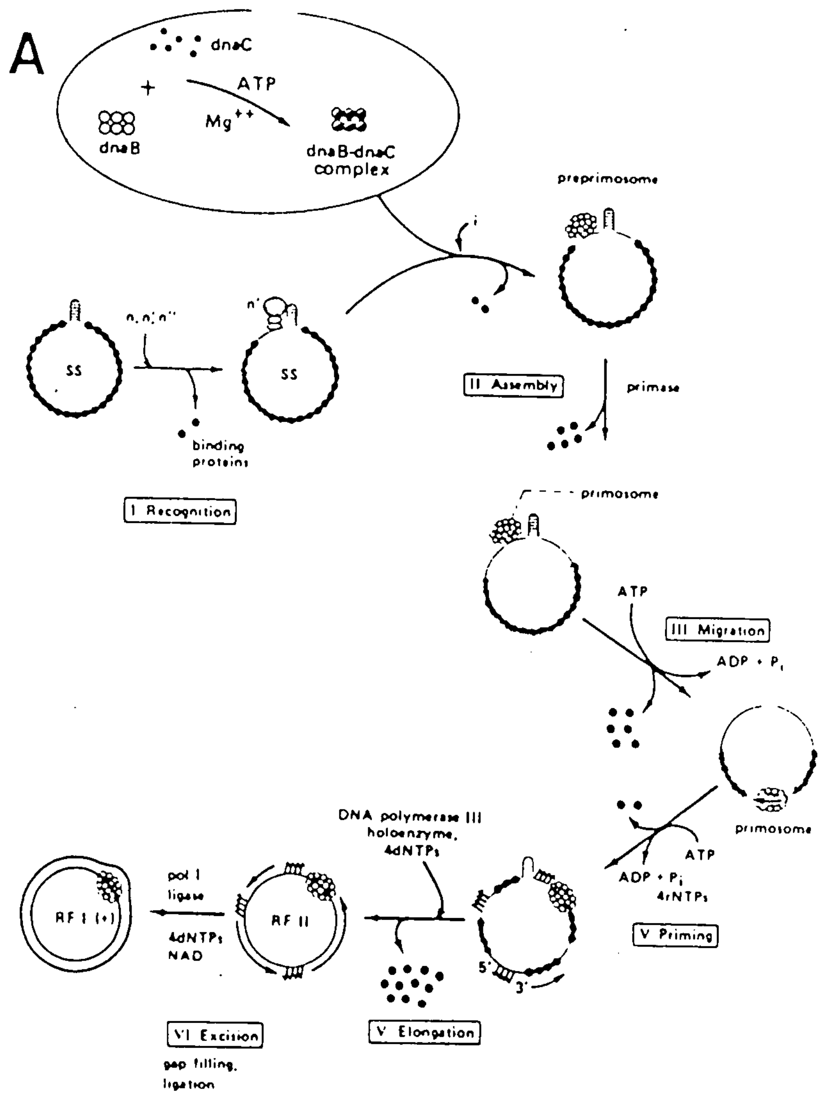
start strands de novo, but can only extend pre-existing ones (Kornberg,1978). DNA unwinding proteins are needed to expose the strands to polymerase action (Arai & Kornberg,1981c) and topoisomerases are needed to release the positive supercoiling resulting from unwinding (Cairns,1963; Gellert,1981).

The first three of these functions: leading strand synthesis, lagging strand synthesis, and unwinding, are thought to reside in a single complex: the replisome. Although the evidence for its existence is largely circumstantial, being based on the fact that many of the factors required for DNA replication are known to interact in vitro or are found to copurify (Wickner & Hurwitz,1975; Arai & Kornberg,1979; McHenry & Kornberg,1977), the fact that the reactions at the replication fork are necessarily both spatially and temporally close is a persuasive argument for such a structure. Furthermore, it is hard to imagine how proteins such as the dnaB product, which are required for elongation (Wechsler & Gross,1971), but which are present in very small quantities in the cell (20 copies per cell in this case (Wickner et al.,1974)), could act if they were not bound as part of some complex that kept them from diffusing away from their site of action. Fig.1.1 shows a diagram of the replisome (Kornberg,1982). This structure may be regarded as being composed of 4 moieties: the leading strand polymerase, the lagging strand polymerase, the primosome, and the unwinding apparatus.

The unwinding apparatus is composed of two types of helicase: 3' - 5' helicases such as the rep protein, which work on the leading strand template (Yarranton et al.,1979), and 5' - 3' helicases which work on the lagging strand template (Yarranton et al.,1979; Kuhn et al.,1979; Abdel-Monem et al.,1983; Arai et al.,1981a; Kornberg,1982). Among these are the n'/gpdnaB complex (Kornberg,1982) (part of the primosome, see below); the recA (Shibata et al.,1981) and recBC (Mackay & Linn,1976) proteins; and 3 other E.coli helicases: helicase I, II, and III, which have no other known role than the melting of DNA duplexes. It is suggested that helicase II, the most abundant of the E.coli helicases, is the most important in chromosome replication (Geider & Hoffmann-Berling,1981). Helicase I is now known to be an F-encoded protein, the traI gene

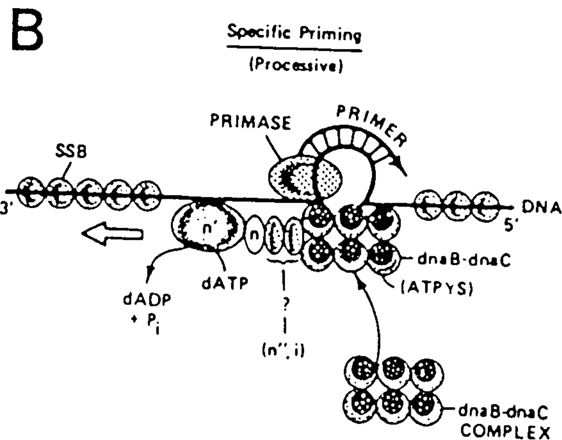
Figure 1.1

A

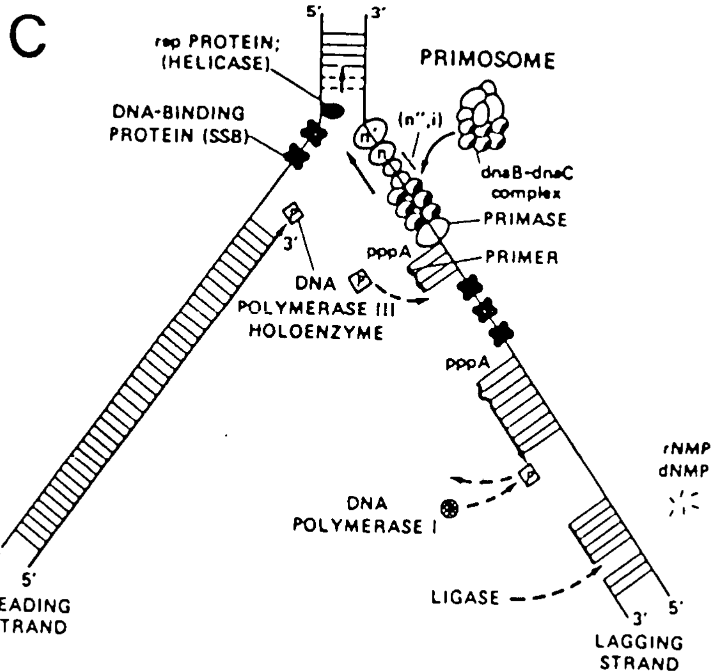


Scheme for assembly and migration of the primosome and the stepwise displacement of SSB in the ϕX SS-RF reaction.

B

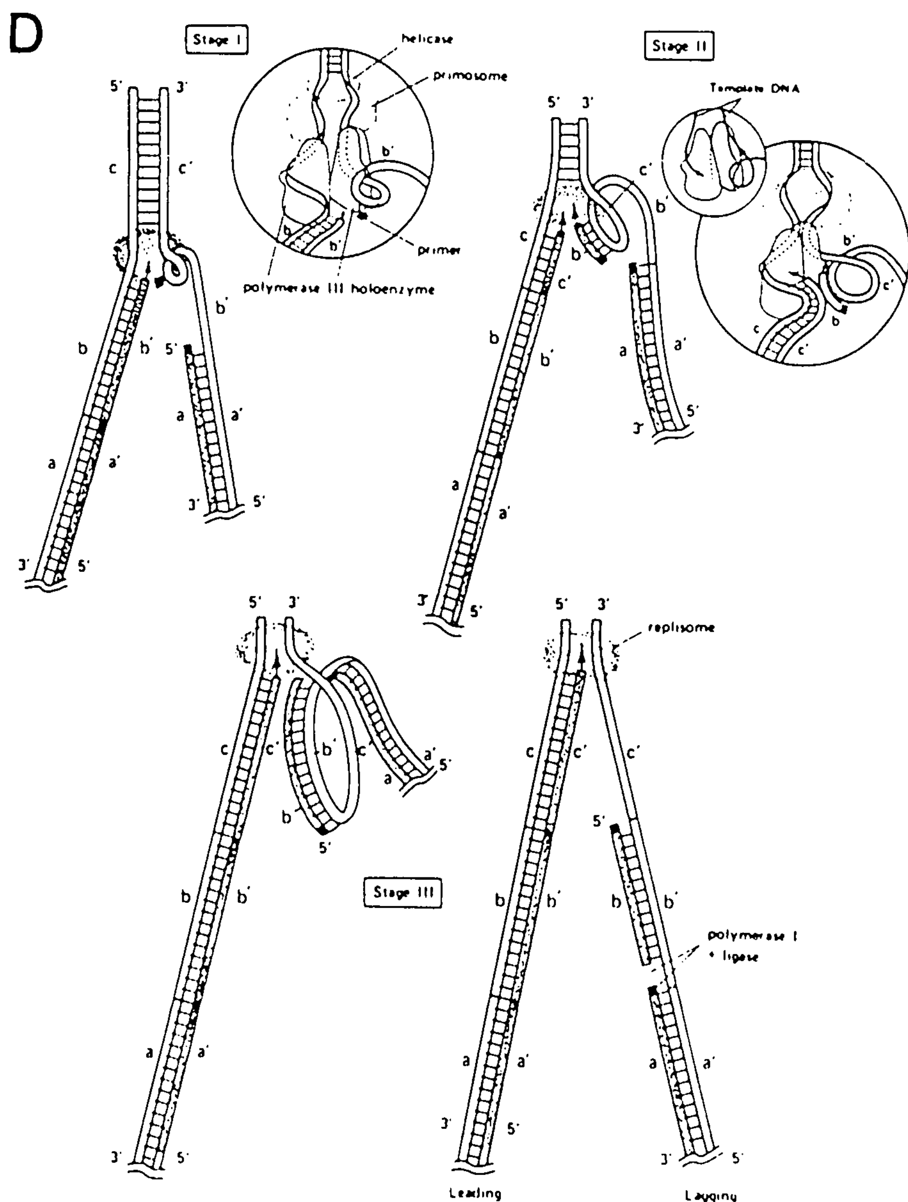


C



Proposed scheme for DNA chain growth at one of the forks of a bidirectionally replicating E. coli chromosome.

D



Proposed scheme for concurrent replication of leading and lagging strands by a dimeric polymerase associated with the primosome and one or more helicases in a "replisome"

Figure 1.1: The replisome

A. Assembly and movement of the primosome on ϕX174 DNA

B. Processive action of the primosome

C. The Replisome

D. Scheme for parallel replication of leading and lagging strands at the replication fork

Reproduced from Kornberg,1982.

product (Abdel-Monem et al.,1983) and probably provides for unwinding of plasmid DNA during conjugation. Helicases are DNA-dependent ATPases which use the energy of ATP to drive the melting of the DNA duplex (Geider & Hoffmann-Berling,1981). Most are processive, going through their cycle of moving into and melting the duplex then hydrolysing ATP, and repeating the cycle indefinitely, without disengaging from the DNA (Geider & Hoffmann-Berling,1981; Kuhn et al.,1979). Helicase II is a notable exception (Kuhn et al.,1979), its great abundance (6000 copies per cell) presumably rendering processive action superfluous. Once the helicases have moved on (or off), the exposed single strands are prevented from reannealing by the binding of single strand binding protein, the product of the ssb gene (Geider & Hoffmann-Berling,1981; Scott & Kornberg,1978; Meyer et al.,1980).

Helicase action has two consequences. One is the exposure of single-stranded DNA which single stranded binding protein stabilises until the polymerases and primase can act, and the other is positive supercoiling in the unmelted DNA. This results from the natural positive twisting of the helix, which the helicases displace, but which cannot be removed without strand breakage. To a certain extent, the native negative supercoiling of the DNA counteracts this, but this is only 0.06 turns per 10bp (Gellert,1981), while helicases will displace 1 turn per 10bp of positive helicity. Only two E.coli enzymes are known to be able to release positive supercoiling: gyrase (topoisomerase II) and gyrase' (topoisomerase II'), a derivative of the latter, in which the 90kd gyrase β subunit is replaced by a 50kd fragment of this (Brown et al.,1979). Topoisomerase II uses the energy of ATP to insert negative supercoils (Gellert et al.,1976), while topoisomerase II' can relax positive supercoils without ATP hydrolysis (Brown et al.,1979), although it is unable to insert negative supercoils (Brown et al.,1979; Gellert,1981). Topoisomerase I, which is also independent of ATP, is inactive against positively supercoiled DNA (Wang,1971).

Polymerisation is carried out by DNA polymerase III (Gefter et al,1971). It is not as yet known how many subunits this enzyme has in vivo. Catalytic function resides in the α subunit, the product of the

dnaF gene (Spanos et al.,1981). The genes that correspond to the three most tightly associated (ε, θ, τ) subunits are not known, but three other polypeptides are coded for by the dnaZ (γ) (Hubscher & Kornberg,1979), dnaX (δ) (Hubscher & Kornberg,1980) and dnaN (β) (Burgers et al.,1981) genes. Temperature-sensitive mutations in any of these genes render the cell elongation-defective. DNA polymerase III holoenzyme has an essentially unlimited processivity (Fay et al.,1981) and it thus seems probable that the leading strand is polymerised in a single unbroken elongation reaction.

The DNA unwinding enzymes, single strand binding protein and polymerase are sufficient to account for the elongation of the leading strand, but the lagging strand requires one further activity, that of the primosome (Kornberg,1982; Arai & Kornberg,1981b). This complex of proteins has the function of moving in a 5' - 3' direction along the lagging strand template, that is, parallel to the elongation of the leading strand, intermittently pausing and synthesising, in the opposite direction, short (1 - 3 bp) RNA oligonucleotides which serve as primers for extension by DNA polymerase III (Arai & Kornberg,1981b).

In all, seven polypeptides are known to be involved in the primosome: primase itself, the product of the dnaG gene, which synthesises the RNA primers (Rowen & Kornberg,1979); the dnaC protein (Weiner et al.,1976; Kobori & Kornberg,1982a); the dnaB protein (Weiner et al.,1976; Kobori & Kornberg,1982b); and the proteins n, n', n'' and i (Weiner et al.,1976), whose genes are not known. In this complex, dnaG protein provides the actual RNA polymerase activity, the n' protein drives the primosome 5' - 3' along the lagging strand template (Arai et al.,1981a), and dnaB protein prompts the intermittent synthesis of primers by altering the configuration of the DNA template (Arai & Kornberg,1981a). Although the other proteins are needed for assembly of the primosome, it is not clear whether they actually form part of its structure, nor whether they impart any enzymic activity to it (Weiner et al.,1976). However, the fact that dnaC mutants may be elongation-defective (Wechsler,1975) strongly suggests that at least this protein is a necessary part of the primosome. It interacts strongly with the dnaB protein (Wickner &

Hurwitz,1975).

The picture of the replisome that emerges is of a complex of synthetic machinery moving together round the replicating chromosome. All the components are seen to move in one direction, save one, the DNA polymerase for the lagging strand. Kornberg (1982) suggests that it might also move in the same direction if the lagging strand is looped around the primosome, such that a polymerase dimer can replicate the leading strand and a loop of the lagging strand in parallel (see Fig 1.1). Without some scheme of this sort, it is difficult to see, when only twenty copies of DNA polymerase III holoenzyme per cell occur (McHenry & Kornberg,1977) and it takes as long for a polymerase to initiate extension as it does to polymerise the the next thousand basepairs (Kornberg,1982), how the synthesis of the lagging strand could keep up with that of the leading strand.

The details of the *in vivo* structure, composition, and cellular location for the replisome are not known, nor is it possible to do more than guess at what it might do when, in the due course of bidirectional replication, it meets its sister replisome coming the other way.

The Cloning of oriC

The cloning of *oriC* was a major step forward in understanding the process of initiation of DNA replication, for it made the *E.coli* chromosomal origin into a molecule of manageable proportions.

Three kinds of *oriC* replicons have been isolated: F-primes, λ transducing phage, and *oriC* plasmids.

Two series of *oriC*-containing F-primes have been constructed. Masters (1975), drawing on the known approximate location of the *E.coli* origin of replication (Masters & Broda,1971; Bird *et al.*,1972; Hohfield & Vielmetter,1973; Jonasson,1973; Louarn *et al.*,1974) isolated a series of F-primes carrying the *oriC* region which showed properties (slow growth, filamentation and reduced DNA content) suggestive of incompatibility

between plasmid-born and chromosomal <u>oriC</u>. Masters <u>et</u> <u>al</u>. (1978) isolated a further series of similar F-primes and demonstrated that these plasmids could integrate into the chromosome by <u>recA</u>-independent recombination at a site indistinguishable from <u>oriC</u>. Hiraga (1976) selected F-primes (F<u>poh</u>+: <u>p</u>ermissive <u>o</u>n <u>H</u>fr) carrying the <u>oriC</u> region that could be maintained in Hfr strains, which normally repress the replication of F plasmids (Saitoh & Hiraga,1975). These plasmids were subsequently shown to be capable of replication in <u>mafA</u> strain, which are defective in F replication (Wada <u>et</u> <u>al</u>.,1976; Wada <u>et</u> <u>al</u>.,1977).

Direct <u>in</u> <u>vitro</u> cloning of <u>oriC</u> by selection of a recombinant, capable of autonomous replication, between a non-replicating ampicillin resistance fragment and EcoR1 digested <u>E.coli</u> chromosomal DNA was first reported by Yasuda and Hirota (1977). The cloned fragment was found to be the same size as an EcoR1 fragment among the first replicated when initiation mutants are allowed to resume DNA synthesis (Marsh and Worcel,1977). Diaz and Pritchard (1978) performed similar experiments to those of Yasuda and Hirota (1977) and, in addition to the 9.8kb <u>oriC</u> EcoR1 fragment isolated by Yasuda and Hirota, isolated a second origin, <u>oriJ</u>, the origin of replication of the cryptic prophage <u>rac</u> (Diaz <u>et</u> <u>al</u>.,1979). By construction of deletion derivatives of <u>oriC</u> plasmids, precise localisation and sequencing of <u>oriC</u> was made possible (Sugimoto <u>et</u> <u>al</u>.,1979; Meijer <u>et</u> <u>al</u>.,1979; Oka <u>et</u> <u>al</u>.,1980).

Shortly after the <u>in</u> <u>vitro</u> cloning of <u>oriC</u> was reported by Yasuda and Hirota, von Meyenburg <u>et</u> <u>al</u>. (1978a) and Miki <u>et</u> <u>al</u>. (1978) reported isolation of λ<u>asn</u> transducing phages which were unique among λ transducing phages in being able to superinfect a homoimmune <u>recA</u> λ lysogen. The DNA of these phage was present as plasmid in host cells; the phage contained <u>oriC</u> and was used as a source of DNA for construction of <u>oriC</u> plasmids indistinguishable from those constructed by Yasuda and Hirota (1977). Construction, from a λ<u>asn</u> transducing phage, of an autonomously replicating deletion derivative, pCM959, which carried only chromosomal DNA (Meijer <u>et</u> <u>al</u>.,1979), finally allayed any fears that replication of <u>oriC</u> plasmids might be a phenomenon caused by interaction between chromosomal DNA and foreign sequences in the various <u>oriC</u>

constructs.

Plasmids carrying oriC as their only replication origin proved very difficult to propagate. This is in part due to their (usually) low copy number and inherent instability (Messer et al.,1978); but also due to the ability of many oriC plasmids to integrate into the chromosome via a recA-independent recombination event (Masters et al.,1978), and the fact that this is favoured by the deleterious effect that these plasmids have on cell growth when they are present in the autonomous state, probably as a result of overexpression of the atp operon (von Meyenburg et al.,1984).

To avoid this difficulty subsequent in vitro manipulation of oriC has tended to use hybrid replicons in which oriC has been cloned into ColE1-based cloning vectors such as pBR322 or pMK2004 (Oka et al.,1980; Zyskind & Smith,1980; Cleary et al.,1982; Oka et al.,1982). The approach to cloning here is not different from that used for selection of oriC recombinants carrying a non-replicating antibiotic resistance fragment except that in this case a PolA- host is used to screen or select Ori+ recombinants, since ColE1 replicons are unable to replicate in such backgrounds. This allows amplification of the plasmid and facilitates the handling of Ori- derivatives as host-conditional mutants. OriC+ recombinant plasmids will be able to replicate in both PolA+ and PolA- hosts, and OriC- plasmids will replicate in PolA+ hosts but not in PolA- hosts. A third class of plasmid, those able to transform PolA-, but not PolA+, hosts is also sometimes encountered, and this is due to a "high copy lethal" (Hcl) phenotype. Because of the deleterious effects previously mentioned, oriC plasmids which carry atp genes may be unable to reach the high copy numbers characteristic of ColE1 replicons in PolA+ backgrounds without killing their hosts. Such behaviour has been noted by Harding et al. (1982) for plasmids carrying oriC and the atp operon of E.coli, Klebsiella pneumoniae or Enterobacter aerogenes. A similar effect was observed with plasmids carrying the Vibrio harveyi origin region, although in this case the Hcl phenotype did not appear to correspond to the presence of atp genes (Zyskind et al.,1983).

The Phenomenology of Initiation of DNA Replication

Much of our understanding of the process of initiation comes from studying the effects of blocking various steps by using antibiotics or various conditional mutations.

Elongation can be blocked by deoxyribonucleotide analogues (Cozzarelli,1977; Kornberg,1978; Hirose et al.,1983; Gale et al.,1981), DNA damage (Caillet-Fauquet et al.,1977; Rupp & Howard-Flanders,1968), inactivation of a mutant gene product required for this process (Carl,1970; Wechsler & Gross,1971; Sakakibara & Mizukami,1980; Henson et al.,1979), or starvation for DNA precursors (Barner & Cohen,1956; Pritchard & Lark,1964). Characteristically, strains treated in this way show a "rapid stop phenotype" where synthesis of DNA (as measured by incorporation of radioactive thymine into acid-insoluble material) ceases immediately and cell division ceases 20 minutes later (Helmstetter & Pierucci,1968). Two factors may contribute to the inhibition of cell division: induction of the SOS regulon (see below under stable DNA replication), one of whose effects is to prevent cell division (Little & Mount,1982); and the dependence (Jones and Donachie,1973) of cell division on the synthesis of a termination protein, which triggers cell division, during the termination event. The small amount of residual division that occurs after inhibition of elongation is due to cells that have terminated their chromosomes and are in the 20 minute D period of the cell cycle, between termination and cell division (Helmstetter & Pierucci,1968).

Elongation is not affected by inhibition of protein or RNA synthesis (Lark et al.,1963; Maaloe & Hanawalt,1961). This is in marked contrast to initiation which requires both and can be blocked with chloramphenicol (Billen,1959) (which inhibits protein synthesis), rifampicin or streptolydigin (both of which inhibit RNA synthesis) (Lark,1972; Zyskind et al.,1977), or by inactivation of one of the gene products specifically required for this process (Beyersmann et al.,1974; Wada & Yura,1974; Carl,1970; Wechsler & Gross,1971). RNA synthesis has been shown to be part of the process of initiation at oriC in vitro (Kaguni & Kornberg,1984). The

requirement for RNA synthesis at initiation is thought to reflect the need for an RNA polymerase-synthesised transcript for priming, rather than one synthesised by dnaG primase, but since extension of the hypothetical oriC transcript into DNA still remains to be demonstrated, it is still possible that the ori transcript promotes the formation of a secondary structure at oriC propitious for initiation. The role of protein synthesis is considered to be in the formation of an "initiation protein" whose accumulation signals that the cell has reached initiation mass (Helmstetter et al.,1968).

Since these treatments do not affect elongation, DNA replication continues until all replication forks have terminated. Thus, inhibition of initiation results in a "slow stop phenotype" (residual DNA synthesis) (Maaloe & Hanawalt,1961); the amount of residual synthesis depending on how fast the cells are growing and thus how many replication forks are present on a chromosome. It is maximal when cells are growing at their minimum doubling time of 20' when residual synthesis gives an 86% increase in DNA. However, at very slow growth rates, where the majority of chromosomes are not being replicated at all, the amount of residual synthesis will approach zero. In general, the amount of residual synthesis is given by the formula:

$$\left\{\frac{a.\ln 2.2^a}{2^a - 1} - 1\right\} \times 100\%$$

(Martin,1970; Yoshikawa & Sueoka,1963) where a = replication (C) time/doubling time (τ): The time taken to complete it is equal to the time taken to replicate the chromosome (C time).

Blocking of initiation of replication does not seem to induce the SOS response, for cell division continues in initiation mutants at restrictive temperatures (Hirota et al.,1968; Wada & Yura,1974) and λ prophage is not induced (Monk & Gross,1971) (though, naturally, blocking protein or RNA synthesis blocks cell division directly). The result is that initiation mutants make enucleate cells (Hirota et al.,1968).

Accumulation of Initiation Potential

Initiation mutants, when grown at non-permissive temperatures, accumulate "initiation potential" (Eberle et al.,1982a). That is to say, when returned to permissive temperatures they have the ability to initiate several rounds of DNA replication without the need for further protein synthesis. This implies that initiation protein is synthesised and accumulated as the cells grow, and also that initiation protein can be synthesised without the active participation of any of the initiation genes tested, dnaA (Gross,1972), dnaC (Gross,1972), dnaP (Wada & Yura,1974), and dnaB (Zyskind et al.,1977). However, it is possible that accumulation of the reversibly inactivated gene products in dnaA, dnaC, dnaP or dnaB mutants actually furnishes the accumulated initiation potential. The behaviour of strains carrying the dnaA508 allele, whose product is irreversibly denatured at 42°C (Eberle et al.,1982b), suggests that the dnaA protein is one of the initiation proteins, since such mutants require long periods of protein synthesis after being returned to a permissive temperature in order to realise their initiation potential. This is actually a rather surprising result in view of the fact that the dnaA gene is autoregulated (von Meyenburg et al., 1984) and thus that rapid accumulation of dnaA protein is expected after shift-down. A possible explanation of this would be continued autorepression by the dnaA508 product in spite of its irreversible inactivation as an initiation protein.

Eberle et al. (1982b) suggest that the delay in recovery of initiation potential in dnaA508 mutants is due to a periodic requirement or periodic synthesis of dnaA protein, although their results might also be taken to mean that the denatured dnaA508 product exerts a spoiler effect; sequestering other initiation genes, the replication origin, or its own gene.

Accumulation of initiation potential has allowed the design of "shift-block" experiments, where a culture that has been allowed to accumulate initiation potential is returned to a permissive temperature

either before or after the addition of inhibitors of RNA or protein synthesis. Fig 1.2 shows the results of two experiments of this sort (Zyskind et al.,1977). A dnaAts mutant is grown at 30°C in ^{3}H thymine in order to fully label its DNA. On shift to 42°C, the characteristic slow stop of DNA synthesis is seen as ongoing rounds of replication proceed to the terminus. Cell growth continues after DNA synthesis has ceased, and during this period initiation potential accumulates. After a few mass doublings at 42°C, chloramphenicol or rifampicin is added and the culture returned to 30°C. In the chloramphenicol-treated culture, growth ceases but DNA synthesis resumes and duplicates several fold. However, in the rifampicin-treated culture, no resumption of DNA synthesis occurs. Such experiments have been much used in an effort to order the various steps in initiation (Zyskind et al,1977; Zyskind & Smith,1977; Wada & Yura,1974). The above example implies that the necessary protein synthesis step can occur before and independently of the action of the dnaA gene product, but that the RNA synthesis step cannot occur without prior or concomitant dnaA action.

A basically similar experiment has used a cold sensitive/heat sensitive double mutant strain (dnaAts,dnaCcs) in hot/cold or cold/hot shifts to demonstrate that dnaA must act before dnaC. That is to say, when the shift is from conditions permissive for dnaC and non-permissive for dnaA to conditions permissive for dnaA and nonpermissive for dnaC, no initiation occurs, while if the reverse shift is made, initiation can occur (Kung & Glaser,1978). The result is summarised below:

Phenotype before shift	Phenotype after shift	Replication?
DnaA-,DnaC+ (41°C)	DnaA+,DnaC- (20°C)	no
DnaA+,DnaC- (20°C)	DnaC+,DnaA- (41°C)	yes

Figure 1.2

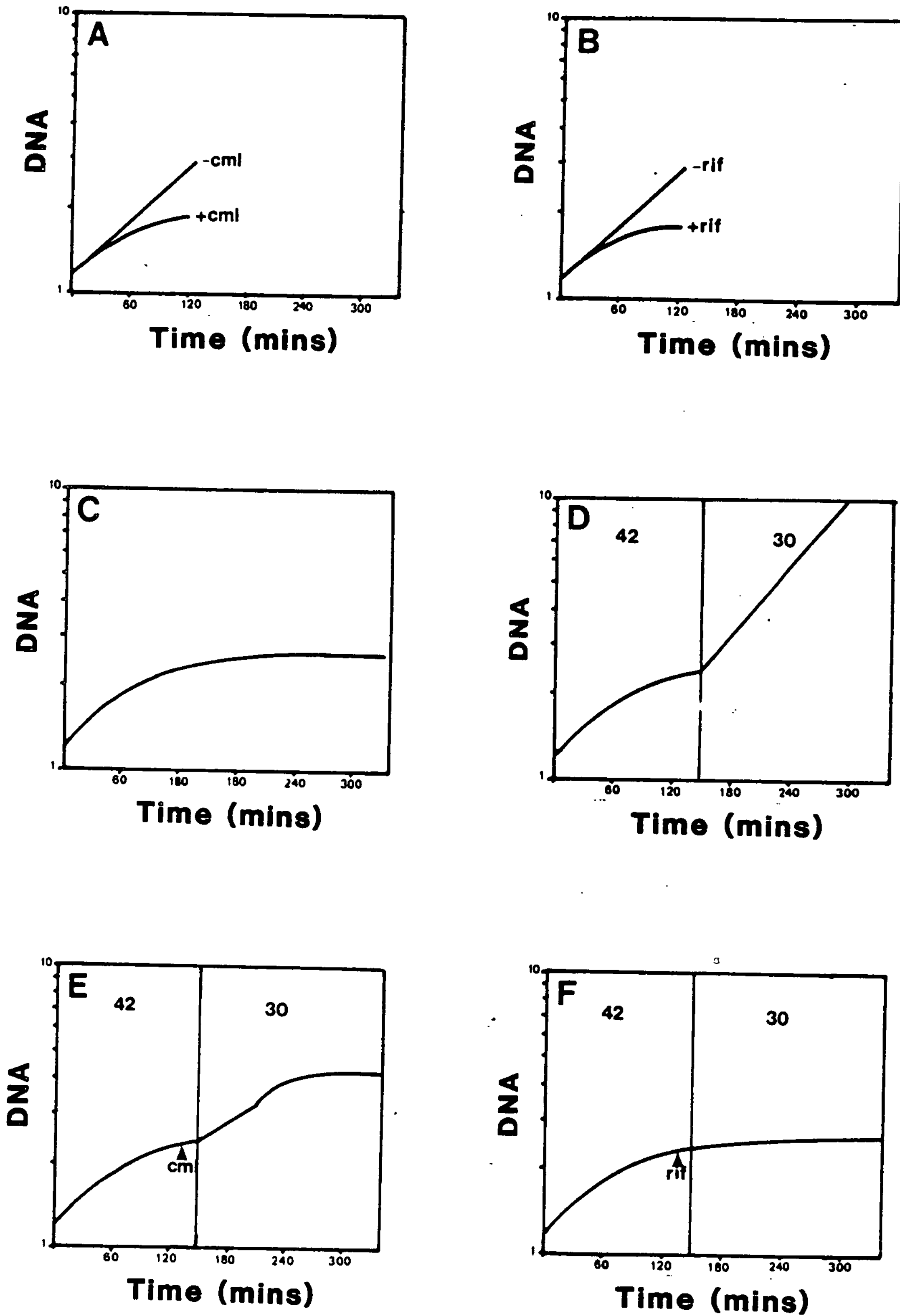


Figure 1.2: Effects of temperature shift and treatment with antibiotics on DNA synthesis in PC236 dnaA5

A. Chloramphenicol added at T=0.

B. Rifampicin added at T=0.

C. Shift to 42°C at T=0.

D. Shift to 42°C at T=0, return to 30°C at T=150.

E. As D, but chloramphenicol added at time indicated by arrow.

F. As D, but rifampicin added at time indicated by arrow.

After Zyskind *et al*, 1977.

It is possible that elongation mutants also accumulate initiation potential, but in this case,the phenomenon might be masked by induction of the SOS response (Schuster _et_ _al_.,1973), and thus of stable DNA replication (Kogoma & Lark,1975).

Stable DNA Replication

E.coli cells, when subjected to treatments that either damage DNA (such as ultraviolet irradiation, treatment with mutagens such as MMS, or exposure to nalidixic acid) or prevent elongation (such as thymine starvation), induce a complex of activities collectively known as the SOS response (Radman,1975; Little & Mount,1982). Two proteins are paramount in the control of the SOS response: the _recA_ protein and the _lexA_ repressor. SOS-inducing treatments activate the _recA_ protein causing it to catalyse the autolysis of the _lexA_ repressor (Little,1984) and thus derepressing the genes of the SOS regulon. Prominent among these genes are those involved in the repair of damaged DNA and in recombination (including the _recA_ protein itself). The products of other genes of the SOS regulon repress cell division.

The SOS response also induces a form of DNA replication known as inducible stable DNA replication (iSDR) (Kogoma _et_ _al_.,1979). Unlike normal DNA replication, this is capable of indefinite continuation in the absence of protein synthesis. Closely similar to iSDR is constitutive stable DNA replication (cSDR) which occurs in strains with an inactive gene for RNaseH (Ogawa _et_ _al_.,1984; Torrey _et_ _al_.,1984; Kogoma _et_ _al_.,1985 in press). Although I shall discuss these under the same heading, the two processes do differ in two respects: Inducible SDR is resistant to UV irradiation and is also mutagenic (presumably error-prone) (Lark _et_ _al_.,1981) while cSDR is neither mutagenic, nor UV-resistant (Kogoma _et_ _al_.,1985 in press).

Both iSDR and cSDR depend on the presence of an active _recA_ product (Lark _et_ _al_.,1981; Kogoma _et_ _al_.,1985 in press). In iSDR this is

required for induction and also for prolonged replication. Data presented in Kogoma et al. (1985 in press) and in Lark et al. (1981) suggest that in both iSDR and cSDR recA is required in initiation, rather than elongation, since replication is slow to stop in a recA(Ts) strain. This result might also be explained if the recA protein is required in some late stage in replication, such as the resolution of complex structures caused by the convergence of replication forks from multiple origins (see below). Normal replication is independent of the recA protein, but requires both oriC and the dnaA protein. dnaA protein is not required for cSDR (Lindahl & Lindahl,1984; Torrey et al.,1984) nor does this pathway recognise oriC as a replication origin, since oriC plasmids cannot replicate in dnaA,rnh strains (Kogoma & von Meyenburg,1983). iSDR is also dnaA-independent (Ciesla & Jonczyk,1980) though it is dependent on dnaE, dnaG, dnaC and dnaB (Kogoma & Lark,1975). It is not as yet known what replication origin(s) is recognised in iSDR.

Four genes have a specific involvement in stable DNA replication: recA (Kogoma et al.,1985 in press; Lark et al.,1981), rnh (Torrey et al.,1984), dnaT (Lark et al.,1978) and sdrT (Lark et al.,1981).

The involvement of recA has been mentioned above. A recessive suppressor mutation, rin has been identified which obviates the need for an active recA gene in cSDR (Torrey & Kogoma,1982). This maps to the 84-89 minute region of the E.coli chromosome. A number of genes relevant to DNA synthesis also map to this region, including oriC, rep, dnaP, and polA (Bachmann,1983), as well as the dnaA suppressor mutations dasB, dasC (Atlung,1981).

As has been mentioned above, rnh mutants are constitutive for stable DNA replication and independent of oriC and the dnaA product, although these are used when available (DeMassey et al.,1984). The dasF class of dnaA suppressor mutations (Atlung,1981) are also rnh mutations, as are the sdrA mutations (Kogoma et al.,1981). All three classes share the same phenotypes and both in vitro (Ogawa et al.,1984) and in vivo (De Massey et al.,1984) studies indicate that this results from the fact that

RNaseH is needed for discrimination between _oriC_ and other potential replication origins. Replication in _rnh_ mutants starts from 4 or 5 identifiable origins other than _oriC_ (DeMassey _et al._,1984) two of which are very close to the replication terminus at 32 minutes, the others mapping at 95 minutes, 45 minutes and 20 minutes. In the _in vitro_ initiation system, omission of RNaseH allows the recognition of substrates other than _oriC_ and the reaction is no longer dependent on the _dnaA_ protein (Ogawa _et al._,1983)

A second class of mutants constitutive for stable DNA replication are the _sdrT_ mutants (Lark & Lark,1980). As replication in these strains is mutagenic, they have been judged to be constitutive for inducible stable DNA replication. The mutations map at 99 minutes, very close to _dnaC_, and may be allelic with the _dnaT_ mutations (see below). Like _rnh_ mutations, _sdrT_ mutations suppress the temperature-sensitive DNA synthesis defect of _dnaA_ mutants, in the sense that _dnaAts_, _sdrT_ strains are able to synthesise DNA at high temperatures in the absence of protein synthesis and without inducing treatments. However, it has not been reported whether these mutations can render _dnaAts_ mutants temperature-resistant for growth. In this context, it is interesting to note that a _dnaA_-suppressor mutation, _dasG_ (see below) maps very close to _sdrT_ (Atlung,1981).

Unlike _rnh_ mutants, _sdrT_ mutants are unable to carry out the normal _recA_-independent DNA replication, and are thus completely dependent on the presence of an active _recA_ product (Lark _et al._,1981). Like _rnh_ mutants, _sdrT_ mutants show a slow stop of DNA synthesis when a temperature-sensitive _recA_ product is inactivated by temperature shift (Lark _et al._,1981). _In vitro_ experiments on crude lysates of _sdrT,recA_ts strains showed that the active _recA_ protein can complement the temperature-sensitive replication of _sdrT,recA_ts strains, and that this complementation is inhibited by ATP-γ-S (Lark _et al._,1981), which inhibits the recombinational activities of _recA_ while stimulating its "protease" function (Craig & Roberts,1980).

In contrast, dnaT mutants are unable to carry out stable DNA replication and thus, in such strains, chloramphenicol causes a slow stop of DNA synthesis regardless of whether they have been exposed to inducers of the SOS response (Lark et al.,1978).

Inducible stable DNA replication is error-prone, and is induced not only by SOS system inducers, but also by a nutritional shift-up (Lark & Lark,1978). This has led Lark et al. (1981) to suggest that it serves to enhance the mutation frequency in fluctuating environments. They argue that when cells shift from a poor to a rich environment mutagenic stable DNA replication forks will be established which will be diluted out by normal oriC/dnaA-dependent forks if the rich environment persists but if poor conditions soon return, many cells will still have mutagenic stable replication forks, that will allow the accumulation of potentially adaptive mutations.

Lark et al. (1981) propose a model for the mechanism of iSDR, in which, under normal circumstances, dnaT product is incorporated into the replisome at initiation and remains a part of it until termination, when it is required for disassembly of the replisome. They suggest that induction of stable replication causes the recA protein to catalyse the cleavage of the dnaT product and substitute for it in the replication apparatus, giving rise to a replisome which is no longer able to disassemble at termination. In this scheme, dnaT mutants are seen as being "recA-resistant".

Initiation Mutants

dnaP

The single dnaP mutant strain, KY2750, has recently been reinvestigated, and the results cast doubt on the classification of dnaP among the initiation genes.

KY2750 was originally isolated as a phenethyl alcohol resistant mutant and found to be temperature-sensitive for DNA synthesis, showing the slow stop of DNA synthesis characteristic of initiation mutants. Return of cultures to permissive temperature after rounds of replication had been completed allowed immédiate resumption of DNA synthesis, indicating that the mutant gene product was reversibly inactivated; resumption of synthesis was not prevented by addition of chloramphenicol or rifampicin, indicating that dnaP was not needed for synthesis of other initiation proteins, and that the rifampicin-sensitive step in initiation of replication could proceed in its absence (Wada & Yura,1974).

KY2750 was resistant to phenethyl alcohol and hypersensitive to deoxycholate, and since both these substances exert their effect primarily at the cell membrane (Silver & Wendt,.1967), it seemed likely that the dnaP product allowed for some essential association between the cell membrane and the initiation apparatus. Nonetheless, no conspicuous alteration of the complement of membrane proteins could be observed on shift of KY2750 to restrictive temperature, except for the disappearance of a 40kd polypeptide, an effect that is common to many treatments that block DNA synthesis (Inouye & Pardee,1970; Ladzunski & Shapiro,1973).

dnaP was reported to map between metE and cya (Wada & Yura,1974). Recently KY2750 has been reinvestigated, and found to carry a temperature-sensitive mutation in dnaG, dnaG2903, (Murakami et al.,1985). The authors report that a second temperature-sensitive mutation exists in KY2750, together with a suppressor of dnaG2903, but the temperature sensitivity could not be cotransduced with ilv, as would have been expected for a mutation mapping to the dnaP locus. Furthermore, they report an inability to isolate initiation mutants by localise mutagenesis of the dnaP region.

Murakami et al. (1985) do not specifically state that dnaG2903 mutants are initiation-defective, though they do say that they are defective in DNA synthesis at non-permissive temperatures; nor do they

report whether phenethyl alcohol resistance accompanies the dnaG2903 mutation. If dnaG2903 is indeed initiation-defective, then dnaG joins dnaB and dnaC among primosome components with initiation-specific functions.

dnaI

Very little is known about the dnaI gene beyond its existence, its map position at around 40' on the 1983 genetic map (Bachmann 1983; Beyersmann et al.,1974; Wechsler,1978) and the fact that it shows the slow stop phenotype typical of initiation mutants (Beyersmann et al.,1974).Its map position close to the replication terminus may be significant (the other initiation mutants map within 10' of the origin of replication). Wechsler (1978) reported difficulty in mapping this mutant by transduction, so it seems possible that it is not a single mutation.

dnaA

The dnaA gene is the most intensively studied of all the initiation genes. A substantial collection of mutants has been accumulated including cold-sensitive (Kung and Glaser,1977), amber (Schaus et al 1981a), and transposon insertion mutants (Kogoma and von Meyenburg,1983); all in addition to the many heat-sensitive mutations (Beyersmann et al.,1974; Carl,1970; Sevastopoulos et al.,1977; Wechsler and Gross,1971; Hirota et al.,1970; Abe and Tomizawa,1971). The cold-sensitive and heat-sensitive mutants all show a slow stop phenotype on shifting to non-permissive temperatures indicating that dnaA has no part in the elongation process.

Of all the initiation genes dnaA has received the most detailed and sustained attention, partly because it alone has been found to be specific for oriC (Marsh & Worcel,1977; von Meyenburg et al.,1979) (in

fact, pSC101 is also dnaA dependent (Felton et al.,1979) but no other known plasmid or phage requires this gene for replication (Nishimura et al.,1971; Tresguerres et al.,1975; Nishimura et al.,1973; Moody & Runge,1972; Goebel,1973; Goebel,1974; Lindahl et al.,1971; Chesney & Rothman-Scott,1978)). In contrast, dnaC is required for the replication of plasmids of the F2 and I2 incompatibility groups (Goebel,1973). The status of dnaI and dnaP is not clear. Both can be integratively suppressed by the F plasmid (Beyersmann et al.,1974; Wada & Yura,1974) but this may reflect an effect of the B/r versus K-12 genetic backgrounds (Wechsler,1978). dnaA thus qualifies as a replicon-specific initiation gene.

dnaA is the first gene in a cluster of four genes of DNA metabolism at 82' on the genetic map of E.coli some 50kb from oriC (von Meyenburg et al.,1980). These are all transcribed in the same direction (outwards from oriC) in the order dnaA-dnaN-recF-gyrB (Bachmann,1983). dnaN codes for the β subunit of DNA polymerase III holoenzyme (Burgers et al.,1981), gyrB codes for the novobiocin-sensitive β subunit of DNA gyrase (Gellert,1981), and recF for a 40.5kd protein involved in recombination and the repair of damaged DNA (Blanar et al.,1984). It is possible that these genes are cotranscribed, but it appears that all have their own promoters, and there is no evidence for the existence of a polycistronic message except in the case of dnaA & dnaN (Sako & Sakakibara,1980). Tn10 insertions in dnaA do not abolish the expression of "distal" genes (Kogoma & von Meyenburg,1983), but Tn3 insertions in dnaA reduce but do not abolish dnaN expression, indicating that dnaA and dnaN are normally cotranscribed, but the dnaN gene has a promoter of its own (Sako & Sakakibara,1980).

Complete sequence data for dnaA and the neighbouring recF, dnaN and rimF genes is now available (Hansen et al.,1982; Blanar et al.,1984) confirming that the dnaA product is a mildly basic protein of 52.2kd. It is a DNA binding protein and has a preference for oriC and other sequences containing a close match to the sequence TGTGGATAA (Fuller & Kornberg, 1983), but this is all that is known about its biochemical action. The evidence accumulated to date indicates that dnaA acts at

the level of transcription. It controls the transcription of its own gene and the trp and phe operons (Atlung et al.,1985, in press; Atlung & Hansen,1983). The presence of promoters in and around oriC which are associated with dnaA binding sites (Lother et al.,1981; Fuller et al.,1984) suggests that it may also control transcription of oriC, a facet of its action that I will expand on in a later section.

dnaA mutations appear to interact with the sdrT and dnaT genes or their products, since at 30°C the dnaA508 and dnaA167 mutations suppress dnaT and sdrT mutations, restoring the inducibility of stable DNA replication in dnaT mutants and abolishing constitutive stable DNA replication in sdrT mutants (Lark et al.,1981).

Amplification of the dnaA gene has only a modest effect on the level of dnaA protein in the cell and, using transcriptional and translational fusions, Atlung et al. (1985 in press) have shown this to be due to autoregulation at the level of transcription (although the control of the levels of dnaA RNA by the dnaA protein remains to be demonstrated). Transcriptional and translational fusions with the dnaA gene are repressed in DnaA+ backgrounds and for this to occur, the 9-11 basepair dnaA binding site has to be present This sequence, which also occurs four times in oriC, lies between the two dnaA promoters, dnaA1p and dnaA2p. Since dnaA1p, the upstream promoter, can be removed without any noticeable effect on control of the dnaA gene, the dnaA binding site is presumably an upstream control region for the dnaA2p promoter. dnaA has a leader sequence of some 120bp (Hansen et al.,1982) though it is not clear what role if any this may play in control of the dnaA gene.

The close clustering and, in the case of dnaN and recF (Blanar et al.,1984), actual overlap of genes in the dnaA cluster, is very suggestive of coordinate control and there is some evidence to support this (Sako & Sakakibara,1980). It would thus be of great interest to know if the three upstream genes participate in the response of the gyrB gene to supercoiling. This gene is repressed by supercoiling and derepressed by relaxation of its coding DNA (Gellert et al.,1982).

Apart from providing an elegant homeostatic control of supercoiling, this mechanism may allow feedback control of DNA replication. Supercoiling of the chromosome is believed to be segregated into between 20 and 100 independently wound loops (or "domains") separated by anchor points that prevent the transmission of superhelical tension between these domains. This structure is manifested in the phenomenon that a single nick is insufficient to relax the whole of a supercoiled E.coli nucleoid (Worcel and Burgi,1972; Pettijohn and Hecht,1973).

I should like to suggest that any area of a domain (a domain is between 1 and 5 minutes or 40 - 200 kb long) will, as replication proceeds, be subject to cyclical change in superhelical density in four phases.

1) Resting phase: No replication of the domain is in progress. Superhelical density is at the "ground state" level of 5 supercoils per kb

2) Prereplicative tension: A replication fork has entered the domain and the unwinding that goes ahead of it causes positive superhelical tension to accumulate in the unreplicated part of the domain.

3) Relaxation phase: The DNA has been replicated and, having only one anchor point (the other end being the free end at a replication fork), cannot be supercoiled.

4) Recovery phase: The replication of the domain has been completed and both the daughter strands have anchor points. Superhelical density is being re-established.

The dnaA gene cluster is very close to oriC and may occupy the same domain of supercoiling. If so, then the initiation of replication will alter the dnaA cluster's superhelical density to "prereplicative tension" levels, which will probably derepress the gyrB gene and possibly the

other genes of the cluster. One minute later, the cluster will become relaxed as the replication fork passes through it and synthesis will decline somewhat, staying at relaxation phase levels until domain replication is complete, when it will gradually decline to "basal" resting phase levels as the native level of supercoiling is restored. Such an oscillation might serve as a signal that replication had occurred and set in train other cell cycle control processes.

Overproduction of the _dnaA_ protein from a high copy number plasmid has a surprisingly slight effect on cellular physiology, the only obvious consequence being a modest increase in cellular DNA content. Since this is accompanied by an increased origin:terminus marker ratio, it seems that abortive overinitiation takes place, such that replication forks are formed but do not proceed to the terminus (Atlung _et al_.,1985 in press). Therefore, if _dnaA_ does control initiation, its action is evidently subject to checks and balances to prevent overreplication. It would be of some interest to learn where (if at any fixed point) the abortive replication forks terminate, and what their eventual fate may be. Since these results do indicate that _dnaA_ is involved in the control of chromosome copy number, but also suggest that other factors are involved, it seems to me that a thorough study of the other genes involved in initiation of replication is required to set the studies on _dnaA_ in perspective.

dnaC and dnaT

These two genes are tightly linked (Lark _et al_.,1978) and may form a single transcriptional unit. _dnaC_, which codes for a polypeptide of 31.5kd (Kobori & Kornberg,1982a) has long been familiar as an initiation gene. Its role seems to be primarily in the assembly and maintenance of the structure of the replisome since it interacts with _dnaB_ (Wickner & Hurwitz,1975), and some _dnaC_ mutants have an elongation-defective phenotype (Wechsler,1978).

Unlike dnaA, dnaC function is required for the replication of many plasmids and phages (Kranias & Dumas,1974; Collins et al.,1975; Mayer et al.,1977; Hasanuma & Sekiguchi,1979; Womble & Rownd, 1978), and also for stable DNA replication (Kogoma & Lark,1975). Its biochemical activity is unknown but it is a necessary factor in the prepriming step of DNA replication. It acts after the binding of proteins n,n',n''and before the action of the dnaG primase which synthesises the short RNA primers that prime replication by DNA polymerase III during elongation (Weiner et al.,1976). Kornberg (1982) suggests that the primosome is linked to the replisome and moves along with it priming lagging strand synthesis. If dnaC is required only for the assembly of this structure, then its initiation-defective phenotype is easy to understand in that it need only be active when primosome assembly is required (i.e. at initiation). The dnaB protein, on the other hand, with which dnaC interacts, has an ATPase activity which is actually required for the the priming reaction itself (Arai & Kornberg,1981a) so that dnaB mutants are usually elongation-defective (Zyskind & Smith,1977; Bachmann,1983).

As might be expected for a protein whose primary role may be in lagging strand synthesis, the dnaC product appears to act after the transcription event in initiation (Zyskind & Smith,1977) (see below).

The most frequently used dnaC mutation, dnaC2 (Carl,1970) has been found to be accompanied by a closely linked dnaT (Lark et al.,1978) mutation. In the presence of the accompanying dnaC mutation, dnaT2 ameliorates the severity of the DnaC(Ts) phenotype so that dnaC2,dnaT+ is unable to grow at 37°C while this is a permissive temperature for the dnaC2,dnaT2 double mutant. However, when separated from dnaC, the dnaT mutants are temperature-sensitive for DNA replication, and (although this seems to have escaped the authors' notice) have the slow-stop phenotype characteristic of initiation mutants. This would be consistent with the apparent interaction between the dnaA and dnaT products mentioned above under dnaA (Lark et al.,1981). At temperatures permissive for growth and DNA synthesis, dnaT strains make filaments and are unable to carry out induced stable DNA replication,

although it is not known whether the mutation prevents stable DNA replication in backgrounds where stable DNA replication is constitutive such as the sdrA/rnh mutants. The dnaT mutations so far studied are at least partially dominant in cis and in trans (Lark et al.,1978).

dnaB

Many phage and plasmids require the dnaB product for their replication (Dumas,1978; Lanka & Schuster,1970; Goebel,1970), though some, such as the phage P1 (D'ari et al,1975) and the plasmid R1 (Wang & Iyer,1978), can substitute their own analogue. Among the many elongation-defective dnaB(Ts) mutants, it has been found that one, dnaB252, shows the characteristic slow-stop phenotype of initiation mutants (Zyskind & Smith,1977). It is not hard to comprehend this when one considers the vital role that dnaB plays in the priming of the synthesis of the lagging strand. In addition to interacting with the dnaC product and with the i factor to form the second component of the primosome it is dnaB which 'prompts' the dnaG primase, in an ATP-dependent reaction, to initiate synthesis of an RNA primer (Arai & Kornberg,1981a; Wickner & Hurwitz,1975). Thus dnaB is active both in the prepriming assembly reaction and in the priming reaction itself; dnaB252 is presumably specifically defective in the former.

It is rather surprising, therefore, to find that dnaB252 mutants, although they accumulate initiation potential, are prevented from reinitiation by the presence of rifampicin during shift-down (Zyskind & Smith,1977), a treatment which has no effect on reinitiation in dnaC mutants (Zyskind et al.,1977). This suggests that dnaB action is required before or during the RNA synthesis step in initiation. One might expect the dnaB product to act at the same stage as the dnaC product, i.e. after RNA synthesis has been completed. In fact, unless dnaB and RNA polymerase are simultaneously active, no initiation occurs. This is shown by an experiment (Zyskind & Smith, 1977) where a dnaB252 mutant was grown at 42°C to allow accumulation of initiation capacity, and then

returned to 30°C after addition of rifampicin. After 10 minutes at 30°C in the presence of rifampicin, the culture was returned to 42°C in the absence of rifampicin. No initiation was observed. This experiment is shown schematically below:

A	B	C
42°C, -RIF	30°C, +RIF	42°C, -RIF
dnaB inactive	*dnaB* active	*dnaB* inactive
RNA pol active	RNA pol inactive	RNA pol active

If RNA polymerase and the *dnaB* product acted sequentially, this scheme would give rise to initiation, since if RNA polymerase acts before gp*dnaB*, RNA polymerase could act during period A followed by gp*dnaB* action during period B, and if gp*dnaB* acts before RNA polymerase, gp*dnaB* could act in period B followed by RNA polymerase action during period C. The fact that this scheme gives no initiation indicates that the two processes are concomitant. This would indicate that, since *dnaB* is expected to act at the same time as *dnaC* (according to the replisome model), it must act twice during initiation; once during the (early) RNA synthesis step; and again during the replisome assembly step in which *dnaC* is expected to be involved and in which, as part of the replisome, *dnaB* product is an obligatory participant.

An alternative explanation of these results would be that the *dnaB252* product "poisons" the initiation process, preventing RNA polymerase action, in which case some early step in initiation (possibly the RNA synthesis step) would be inhibited by the *dnaB252* product in period A, by rifampicin in period B, and again by the *dnaB252* product in period C.

This latter explanation would be more consistent with the observation (Kaguni & Kornberg,1984) that *in vitro* the RNA synthesis step in

initiation can occur without the participation of any of the components of the initiation apparatus except RNA polymerase.

Suppressors of dnaA

It seems possible that only a fraction of the initiation genes of E.coli have been identified, since only single mutant alleles of dnaP and dnaI have so far been isolated, and dnaP was not isolated by screening for DNA synthesis mutants, but by selection for phenethyl alcohol resistance. (Beyersmann et al.,1974; Wada & Yura,1974). Furthermore, it is clear that many of the gene products involved in initiation, such as DNA gyrase and RNA polymerase, may not yield temperature-sensitive initiation mutants due to the other effects that such mutants will have on cell physiology (Gellert,1981; Kreuzer & Cozzarelli,1979; Wahle et al.,1985; Miller et al.,1976).

For these reasons alone, the study of suppressors of known initiation mutants promises to add considerably to our knowledge of the genetics of initiation. Selection of temperature-sensitive initiation mutants directly is laborious (Beyersmann et al.,1974) and seems to yield a vast preponderance of dnaA and dnaC mutants. By selection of suppressors, we may hope to identify new initiation genes, and having localised them, to selectively mutagenise them in the hope of obtaining conventional temperature-sensitive initiation mutations in these genes.

This is by no means the only reason for this approach. Perhaps the major rationale for attempting the isolation of suppressor genes is to detect the *in vivo* interaction of the various factors involved in a complex process (Jarvik & Botstein,1975).

dnaAts mutants have a remarkably high rate of reversion to temperature-resistance, of which a very small fraction is due to true reversion (Atlung, 1981); the rest being due to extragenic suppressors (Atlung,1981; Wechsler & Zdienicka,1975). At least seven loci can be

identified for these suppressor mutations. In addition, there are five (possibly four) cloned suppressors which were identified during attempts to clone the dnaA gene, but which are actually other genes whose new molecular environment allows them to function as suppressors (Projan & Wechsler,1981; Takeda & Hirota,1982). Overexpression due to high copy number is the most obvious explanation for this behaviour, but Rowen et al. (1982) have reported the isolation of a low copy number suppressor of dnaA cloned in λ. As yet, none of these cloned suppressors has been genetically mapped so we cannot tell whether any of them are synonymous with any of the chromosomal suppressor mutations.

The isolation and characterisation of such suppressors offers a powerful tool with which to analyse interactions between genes. However, it must be stressed that a suppressor can arise in a number of ways, not all of which will necessarily be interesting:

1) Bypass suppression: the archetypal examples of this are the rnh (dasF, sdrA) mutations which cause constitutive stable DNA replication (Kogoma & von Meyenburg,1983), and integrative suppression (Nishimura et al.,1971; Lindahl et al.,1971; Moody & Runge,1972; Nishimura et al.,1973; Goebel,1974; Tresguerres et al.,1975; Chesney & Rothman-Scott,1978) by integration of a dnaA-independent replicon into the chromosome which then drives replication in place of oriC. The characteristic of bypass suppression is that the function of the dnaA gene is completely obviated.

2) Interactive suppression: this may involve direct interaction between the mutant dnaA product and the product of the suppressor or it may be at the genetic level. We know, for instance, that one possible class of mutants would be in a gene that represses the synthesis of the dnaA product, since overproduction of mutant dnaA product can relieve the initiation defect (von Meyenburg et al., 1985, in press). Direct interactive suppression, where a mutant product interacts with another mutant product to produce an active complex, would be the most

instructive type of suppression in terms of the molecular analysis of the process of initiation. Direct interactive suppression is expected to show a degree of allele specificity and where it occurred, one would expect to be able to cross-link the interacting gene products or to identify a specific association between them in vitro.

3) Informational suppression: The classic informational suppressors are the nonsense suppressor tRNAs which insert amino acids at termination codons. However, frameshift (Smith,1979) and missense (Hill,1975) suppressors can also occur, and they may be expected to mimic direct interactive suppressors, in that they will be allele-specific. This class of suppressors is the least illuminating. Their characteristics would be:

a) Very narrow allele specificity, since the suppressor will recognise only two or three of the 61 possible missense codons.

b) A very wide and sporadic range of suppression of unconnected missense mutations.

c) Possible production of an altered mutant gene product due to amino acid substitution.

Although there is no rapid or reliable screen against missense suppressors if a suppressor were to suppress only one out of a closely spaced group of mutations (such as dnaA46, dnaA167 and dnaA5) one would have good cause to suspect informational suppression.

4) Higher order suppressors: interactive and informational suppressors, as discussed above, involve a direct interaction between the suppressor and the dnaA gene or its product. In a system as complex as initiation of DNA replication, it is wise to

keep in mind that a suppressor might act not on the suppressed gene but on one which interacts with it; a mechanism which I shall call second order suppression (indirect suppression). In a system that is very hierarchical, suppression of even higher order may be possible.

In the case of very few of the dnaA suppressors have sufficient tests been applied to distinguish these various forms of suppression. In most cases, only the map position and suppression of a single allele have been reported. The exceptions are dasF (rnh, sdrA), a bypass suppressor (Atlung,1981; Torrey et al.,1984), and rpoB, probably an interactive suppressor (Schaus et al.,1981b; Bagdasarian et al.,1977)

dasA (Atlung,1981)

The dasA mutation maps close to mtl at 80'. This is a rather gene-sparse region containing only nutritional markers so it would seem that dasA is a new gene. The dasA379 mutant is cold-sensitive and has a somewhat raised DNA/protein ratio. This is the phenotype reported for mutants overexpressing the dnaA46 allele (von Meyenburg et al.,1985 in press) (they also acquire temperature resistance). It would seem a reasonable hypothesis that dasA is an interactive suppressor which enhances expression of the dnaA gene. The wild-type product might be a negative regulator of dnaA since the dasA mutation is recessive to wild type.

dasB (Atlung,1981)

The dasB mutations map close to oriC and exhibit a rich-medium-sensitive growth and cell division phenotype. Deletions of the gid gene, adjacent to oriC have a similar phenotype (von Meyenburg & Hansen,1980), although Gid- strains are always rich-medium-sensitive, while dasB strains are sensitive only when combined with a dnaA mutation. sdrA (rnh) mutants are also rich-medium-sensitive (Torrey et

al.,1984). The rich medium sensitivity of *dasB,dnaA46* strains is observed at 33°C, a permissive temperature for the *dnaA46* allele, so it is presumably a consequence of interaction between the 2 mutant products or the mutant *dnaA46* product and the *dasB* locus. Whether this mutation acts *in cis* or *in trans* or is dominant or recessive is not known, which in view of the possibility of its being in *oriC* is unfortunate.

dasC (Atlung,1981)

dasC maps between *ilv* and *rho* and is therefore possibly allelic with the *rep* gene. It is one of the few *dnaA* suppressors for which allele specificity has been reported (see Table 1.1) . The two alleles tested, *dnaA46* and *dnaA204*, are quite widely separated on the *dnaA* gene, and also differ in that *dnaA46* produces a reversibly denaturable product while the *dna204* product denatures irreversibly (Hansen *et al*.,1984).

The allele specificity of *dasC* suggests that the *dasC* product interacts directly with the *dnaA* product. However, it may be that *dasC* causes overexpression of *dnaA* and that *dasC116* does not overproduce *dnaA* sufficiently to suppress *dnaA46*.

rep protein is a helicase which unlike the other known *E.coli* replicative helicases binds to the leading strand template. *rep* mutants are defective in ϕX174 and M13 replication and show a 50% reduced rate of chromosome replication (Kornberg,1982). *rep*- mutants are, however, leaky. It seems probable, however, that in a normal cell the *rep* protein cooperates in or facilitates an unwinding process that accompanies *dnaA* action; we need not assume that it is an absolute essential for this, since *E.coli* has several other of helicases (Geider & Hoffmann-Berling,1981) which might, under most circumstances, substitute for *rep*. It would be interesting to know: (a) whether *rep* mutants are also *dnaA* suppressors, and (b) whether it is possible to isolate null mutants of *rep*.

Table 1.1 : allele specificity of *dasC* mutations

	dnaA46	*dnaA204*
dasC116	cold sensitive, not suppressed	suppressed
dasC382	suppressed	not tested

dasC382 and dasC386 are dominant over wild type; a property one might expect of suppressors that interact directly with the suppressed mutant gene product, since presence of the wild type product of the suppressor gene would not be expected to eliminate the putative active dnaA46/dasC complex. In the case of this mutation we can, on the basis of allele specificity, exclude bypass suppression; and on the basis of dominance, we can eliminate interactive suppression based on the inactivation of a putative repressor of the dnaA gene. The remaining possibilities are that the dasC product interacts with the dnaA product, or that the dasC mutations give rise to excess activity of a positive regulator of dnaA.

dasE (Atlung,1981)

dasE maps between ara and car at approximately 1' and was originally identified as an indigenous suppressor required for the suppressive action of dasG. It is not clear whether it is a dnaA suppressor in its own right, since the only other strain in which its activity has been tested carries a dasC suppressor. It is clear, however, that the combinations dnaA46,dasC,dasE and dnaA46,dasG,dasE are temperature-resistant.

The only gene that appears to be involved in DNA replication in the dasE region is the mafA gene, which is required for the maintenance of the F plasmid (Wada et al.,1976).

dasG (Atlung,1981)

dasG maps close to the dnaC-dnaT cluster and is possibly an allele of one of these genes. The only known mutant allele of dasG appears to require the cooperation of the dasE mutation to suppress dnaA46. It may be recalled that sdrT mutations constitutive for stable DNA replication map to this region (Lark et al.,1981) and it is tempting to

suggest that dasG is likewise an sdrT mutation. This would not necessarily mean that dasG is a bypass suppressor, since the sdrT and dnaA products appear to interact (Lark et al.,1981).

topA

It has recently been shown (Louarn et al.,1984) that topA mutants suppress the temperature-sensitive phenotype of dnaA mutants. The DNA of topA mutants has an increase superhelical density due to the inability of such strains to relax the supercoils inserted by DNA gyrase (Pruss et al.,1982), although topA mutants tend to quickly accumulate mutations in gyrA or gyrB which compensate for this and often ultimately give rise to a reduced superhelical density of the chromosome (Pruss et al.,1982; Dinardo et al.,1982).

Whether this observation actually reflects a direct dependence of initiation on a high level of supercoiling, and a decreased dependence on dnaA activity when the origin DNA is " hypercoiled" remains to be seen since it is almost equally likely that transcription of dnaA, like that of the closely linked gyrB gene (Gellert et al.,1982) is controlled by the level of supercoiling of its DNA and that topA mutants will therefore have increased levels of mutant dnaA protein which has shown to suppress the temperature-sensitive phenotype of dnaA(Ts) mutants.

Evidence that topoisomerase I, the product of the topA gene, has a direct role in initiation comes from the work of Kaguni and Kornberg (1984) which demonstrates that topoisomerase I is a specificity factor in in vitro initiation at oriC, since, in its absence, initiation of replication ceases to be oriC-specific and initiation at oriC ceases to be dnaA-dependent. Thus, it seems likely that topA mutations are a kind of bypass suppressor, though whether they act by allowing the use of alternative origins (as does rnh) or dnaA-independent initiation at oriC remains to be seen.

rpoB

The best studied dnaA suppressor mutations are in rpoB (Atlung,1981; Schaus et al.,1981; Hansen et al.,1984). These mutations are allele-specific for reversible dnaAts mutations: the irreversible dnaA508 and dnaA204 mutations are not suppressed (Hansen et al.,1984). These two mutations map to different ends of the dnaA gene (Hansen et al., 1984). Surprisingly, one of the dnaA amber mutations is also suppressed by these rpoB alleles (Schaus et al,1981b). It is not known as yet where this amber mutation maps, although it seems to give rise to a truncated polypeptide of 33kd (Schaus et al.,1981a) which would place it close to the distal end of the region where the temperature-reversible, rpoB-suppressible mutations map.

In addition to suppression of dnaAts, these mutants are rifampicin-resistant, show increased termination at the trp attenuator, and are permissive hosts for the T7 mutant H3 gene1am342 (Schaus et al.,1981b). The allele specificity of suppressing rpoB alleles rules out the possibility of bypass suppression. Three explanations of their behaviour are currently tenable:

1) The rpoB alleles give a "partial bypass" due to their enhanced transcription termination activity, which substitutes for a necessary termination activity of the dnaA product.

2) The RNA polymerase interacts directly with the mutant dnaA products and stabilises them.

3) The mutant polymerase gives increased transcription of the dnaA gene and thus allows overexpression to compensate for the low activity of the mutant dnaA product

There is a lesson of caution to be learned from the work that has been done on dnaA suppressors. Two of the suppressor mutations,

dasC116 and dasE, are indigenous to genetic backgrounds carrying the dnaA mutations. A similar situation was found with dnaC2 and dnaT2 in the mutant strain PC2 (Lark et al.,1978). In this case, dnaT is apparently a compensatory mutation which enhances the original strain's viability. I anticipate that cryptic das mutations are to be found in other dnaA strains and these may well be affecting our interpretation of the physiology of dnaA and other initiation mutants.

Cloned Suppressors

Attempts to clone dnaA recovered 5 different DNA loci, none of which carries the dnaA gene (Takeda & Hirota,1982; Projan & Wechsler,1981). One of these, sdaA, is the subject of much of this thesis.

Fig. 1.3 shows the restriction maps of of these 5 suppressor genes. Takeda and Hirota have adopted the designation sda for their two cloned suppressors and for the sake of clarity I have tentatively assigned the designations sdaA and sdaB for their clones and sdaC, sdaD, sdaE for the unnamed suppressors reported by Projan and Wechsler (see Table 1.3)

Consideration of the restriction maps of these cloned suppressors (Fig. 1.3) shows that the large EcoR1-HindIII fragments of pSP6 and pYT46 are approximately the same size, and thus these plasmids may be homologous and the sdaB and sdaD genes may be the same. There also exists the possibility that the small HindIII-EcoR1 fragments of pSP6 and pYT47 are homologous, although their small size probably precludes their having any significant coding capacity. sdaC, sdaD, and sdaE are unable to suppress the amber mutation dnaA311, which indicates that these are interactive rather than bypass suppressors. sdaC and sdaE extracts are able to complement dnaA in vitro, but this is not true of sdaD. This result does not distinguish the two possibilities of induced overexpression of dnaA and direct interaction with the dnaA product.

Figure 1.3: Restriction maps of the sda loci

This figure shows restriction maps of the chromosomal inserts in plasmids suppressing DNA. Restriction targets are indicated as follows:

A = AvaI
B = BamHI
b = BglII
E = EcoR1
H = HindIII
h = HpaI
P = PstI
X = XhoI

The shaded portion indicates the approximate position of the sdaA structural gene.

Adapted from Takeda & Hirota, 1982 and Projan & Wechsler, 1981.

Figure 1.3

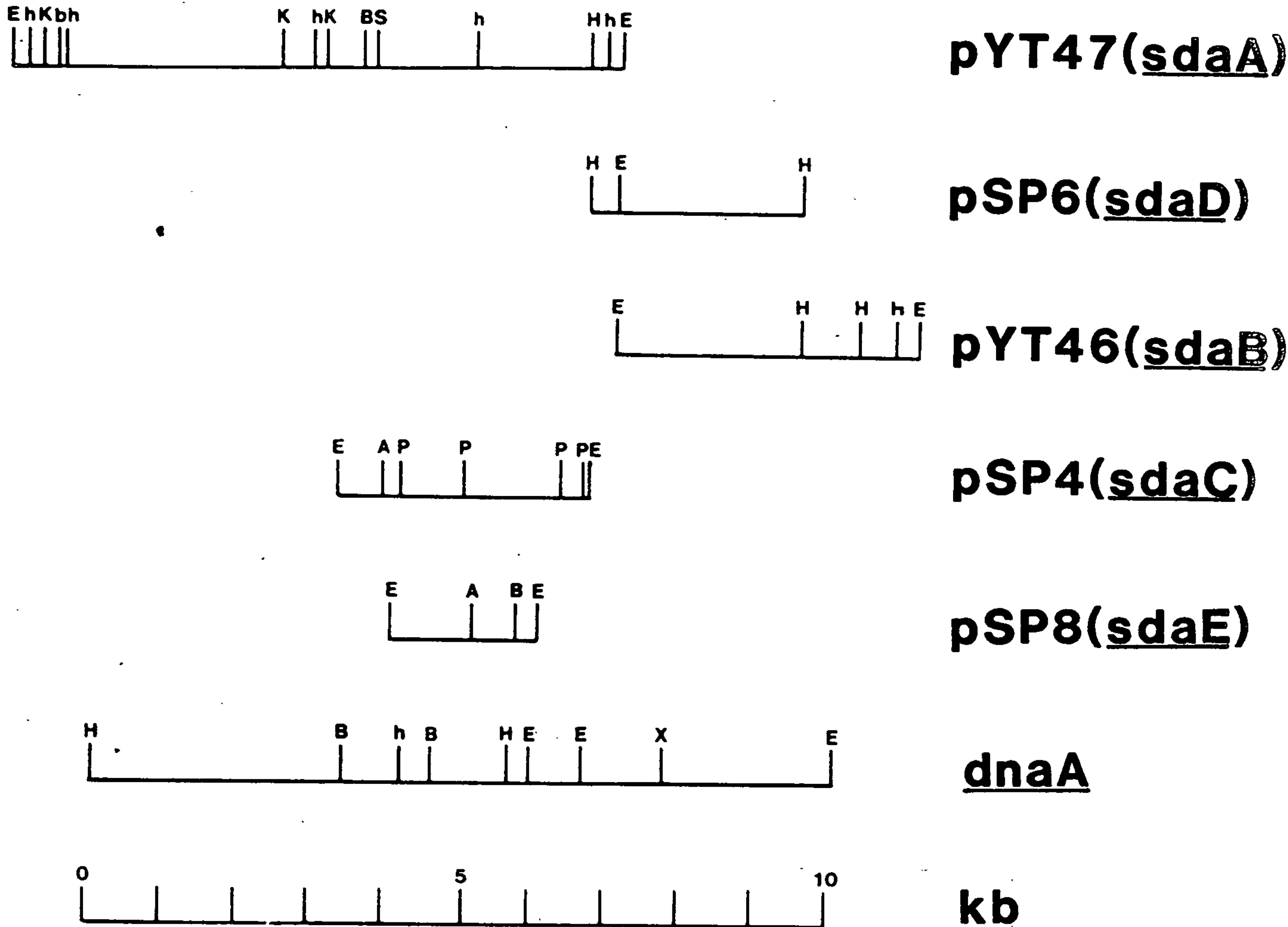


Table 1.3: dnaA suppressors

gene designation	plasmid	reference
sdaA	pYT47	Takeda and Hirota, 1982
sdaB	pYT46	"
sdaC	pSP4	Projan and Wechsler, 1981
sdaD	pSP6	"
sdaE	pSP8	"

sdaA codes for a protein of 68kd, all or part of whose coding sequence is within the bounds of the box indicated in Fig. 1.3. It has a wide allele specificity, suppressing both cold-sensitive and heat-sensitive mutations, although amber mutations have not been tested. It might be a bypass suppressor; but if it induces stable DNA replication it is able to do so without the participation of the recA product since dnaA,recA,sdaA strains are temperature-resistant.

sdaB, like sdaA, gives rise to recA-independent suppression of dnaA and has the same allele range. Neither the nature of the gene product, nor the position of the sdaB gene within the 5.3kb EcoR1 fragment that carries it is known.

pYT46 (sdaB), unlike pYT47 (sdaA), cannot transform dnaA cells to temperature resistance directly, but instead requires several generations at a permissive temperature for establishment of suppression. It is possible that this reflects a requirement for maximal copy number of the plasmid, since ColE1 plasmids cannot replicate efficiently in dnaA cells whose chromosome is not being replicated (Frey et al.,1979).

Some of the sda plasmids have been tested for their ability to suppress other genes, which allows us to eliminate some of the many possible known genes which might be proposed as their chromosomal loci. In other cases, it is possible to eliminate loci by comparison of the restriction patterns. These conclusions are summarised in Table 1.4.

It is natural to consider the other suppressor mutations as potential chromosomal loci for the sda clones. It seems unlikely however that any of the cloned suppressors would correspond to a recessive suppressor mutation. This would eliminate dasA, topA and rnh.

The properties of the various suppressors are summarised in Table 1.5.

Table 1.4 Possible genetic loci eliminated for sda genes

gene	loci eliminated by complementation	loci eliminated by comparison of restriction maps
sdaA	-	dnaA, dnaZ, dnaE
sdaB	-	dnaA, dnaZ, dnaE
sdaC	dnaB, dnaC, dnaZ, rpoB, cya, crp	dnaA, dnaZ, dnaE
sdaD	dnaB, dnaC, dnaZ, rpoB, cya, crp	dnaA, dnaZ, dnaE
sdaE	dnaB, dnaC, dnaZ, rpoB, cya, crp rho	dnaA, dnaZ, dnaE

Restriction data for comparisons were taken from Rowen *et al*.,1982; von Meyenburg *et al*.,1980; Welch & McHenry,1982.

Table 1.5 Suppressors of dnaA

locus	dominance over w.t.	suppression *in trans*	allele specificity	type	possible gene
rpoB	-		+	interactive	
topA	-		?	bypass?	
rnh	-		-	bypass	
dasA	-		?	genetic interactive?	?
dasB	?	?	?	interactive?	*oriC*
dasC	+	?	+	interactive	*rep*
dasE	?	?	?	?	?
dasG	?	?	?	?	*sdrT*, *dnaC* *dnaT*
sdaA		+	-	?	*groE*
sdaB		+	-	?	
sdaC		+	- (not ambers)	interactive	
sdaD		+	- (not ambers)	interactive	
sdaE		+	- (not ambers)	interactive	

A number of mechanisms of suppression have been discussed. These include bypass of the dnaA-oriC pathway, increased expression of dnaA, "complementation" of a missing dnaA function (such as has been suggested for the "terminator rpoB alleles"), modification of oriC structure (topA?, dasC?), and stabilisation of the mutant product by direct interaction. Another possibility is the activation of a "pseudo-dnaA gene", a form of bypass suppression.

Consideration of the possible explanations of dnaA suppression by topA and rpoB mutations suggests that another class might also be considered. I term this "facilitative suppression" and it includes such hypothetical examples as enhanced dnaA action at oriC due to excessive supercoiling in topA mutants, and enhanced termination or transcription at oriC suppressing a reduced dnaA activity in rpoB mutants. Facilitative suppression occurs through an alteration of the structure or the level of a putative substrate of dnaA, enabling more easy dnaA action. However, it should be stressed that if the substrate were the product of the suppressor gene, this would be interactive suppression.

We might thus expect to gain knowledge of the control and biochemical mechanism of action of dnaA as well as the interaction of its product with other gene products. It is therefore disappointing that so few of the dnaA suppressors have been studied in depth, and that suppressors of other initiation genes have not been sought.

DNA Gyrase

Examination of the nucleotide sequence of oriC (Meijer et al.,1979) shows that it has an enormous potential for developing secondary structures and, since negative supercoiling is known to drive conversion of DNA to secondary structures, it would be most surprising if the initiation of replication were not dependent on the activity of DNA gyrase.

Physiological experiments aimed at determining the role of gyrase in the initiation of DNA replication (Zyskind et al.,1977; Orr et al.,1979; Kreuzer & Cozzarelli,1979) have not been particularly illuminating due to the many other effects of the inhibition of DNA gyrase activity, and the fact that the effects of the gyrase inhibitors, nalidixic acid and novobiocin, are rather different to the effects of temperature-sensitive mutations in the gyrA and gyrB genes (Engle et al.,1982; Kreuzer & Cozzarelli,1979; Lockshon & Morris,1983; Orr et al.,1979; Gellert,1981) This is particularly true of naladixic acid, which appears to "jam" DNA gyrase in a complex with DNA which contains a double-stranded nick (Morrison & Cozzarelli,1979), and thus causes very rapid cessation of DNA synthesis (Goss et al.,1965).

DNA gyrase is a tetramer composed of 2 of each of the α (nalidixic acid sensitive, gyrA product) and β (novobiocin-sensitive, gyrB product) subunits. The enzyme inserts negative supercoils into DNA in an ATP-dependent reaction by passing a section of duplex through a double-strand break in the DNA. The result is to insert two negative supercoils in each reaction (Gellert,1981).

RNA synthesis is affected by gyrase since the preference of RNA polymerase for various promoters is altered by supercoiling, as is DNA synthesis and recombination (Gellert,1981).

Orr et al.. (1979) have presented data indicating that gyrB(Ts) mutants are specifically defective in the initiation of DNA replication since the "run-off time" (the time required for cessation of DNA synthesis after inhibition of initiation by chloramphenicol or rifampicin) is not greatly altered at temperatures intermediate between the permissive and non-permissive temperatures, although the overall rate of DNA synthesis is reduced. Nonetheless, the classic slow-stop phenotype is not observed in gyrB(Ts) mutants. A possible explanation of this complex behaviour is afforded by the fact that DNA gyrase exists in at least two forms in E.coli cells: topoisomerase II and topoisomerase II', the latter consisting of gyrase α and a truncated gyrase β subunit (Brown

et al.,1979). It seems possible that topoisomerase II is needed for initiation and topoisomerase II' is needed for elongation, and that the latter is significantly less temperature-sensitive that the former. Since gyrase α is a necessary component of both topoisomerase I and topoisomerase II, this would be consistent with the elongation-defective phenotype of gyrA(Ts) mutants (see below).

In addition to their apparent defect in initiation, gyrB(Ts) mutants also exhibit reduced superhelical density of their nucleoids and this is reflected by a defect in the segregation of nucleoids (Orr et al.,1979; Steck et al.,1984). The result is a change in their observed morphology and a tendency to produce enucleate cells. gyrA(Ts) mutants, on the other hand, seem to be primarily defective in elongation insofar as the results of Kreuzer and Cozzarelli (1979) indicate a decline in the rate of DNA synthesis too rapid to be accounted for by simply stopping initiation. However, the methods of measurement do not really allow comparison between gyrA(Ts) and gyrB(Ts) mutants.

Although the data are rather difficult to interpret, it is probably safe to assume that initiation is dependent on DNA gyrase activity. This is certainly the case with the in vitro initiation assay (Kaguni & Kornberg,1984) where omission of the gyrase β subunit renders the system inactive.

It remains moot, however, whether the dependence of initiation on gyrase activity reflects:

a) A dependence of initiation on supercoiling, as the highly palindromic structure of oriC would suggest (negative supercoiling stimulates the transition of palindromic DNA to cruciform structures (Mizuuchi et al.,1982)).

b) A requirement for active supercoiling during the process of initiation.

c) Some other activity of gyrase during initiation, such as an

interaction with one or more of the other components of the initiation apparatus.

Of these, (a) seems to be at least in part true, since linear substrates are far less active than supercoiled closed circular ones in the *in vitro* initiation assay (Fuller *et al*.,1983). However, even when a supercoiled substrate is used, the *in vitro* reaction is gyrase-dependent, indicating an active role of gyrase in initiation beyond the requirement for *oriC* to be fully supercoiled.

A preferential binding site for DNA gyrase occurs in *oriC* (Lother *et al*.,1984), and coincides with the positions of two closely-spaced *dnaA* binding sites, close to the right-hand boundary of *oriC* (see Fig. 1.4), which is suggestive of an interaction between gyrase and the *dnaA* protein. In contrast to other gyrase binding sites, the oxolinic acid-induced cleavage of DNA that is thought to reflect the supercoiling activity of DNA gyrase (Morrison & Cozzarelli,1979) does not occur at this site, although a number of other sites close to *oriC* are cleaved. Binding to the preferential site is not dependent on supercoiling.

Thus, it seems likely that gyrase is needed in some capacity other than a source of negative supercoiling during initiation of replication at *oriC*.

The Origin of Replication and The Role of Transcription in Initiation.

The smallest stretch of E.coli oriC DNA capable of autonomous replication is between 232 and 245 bp long (Oka et al.,1980). I shall return later to the question of whether other cis-acting sequences outside this stretch of DNA are essential for chromosome replication.

Examination of the nucleotide sequence of oriC (Meijer et al.,1979; Sugimoto et al.,1979) reveals the presence of no less than 24 symmetry elements and a distinct preponderance of the sequence GATC, the recognition sequence for the dam methylase (Razin & Friedman,1982). dam mutants are not defective in initiation of DNA replication (Bale et al.,1979) so it is not clear whether methylation is indeed the important function of these sequences or whether they serve some other purpose. dam methylation is required for the mismatch repair system to distinguish the new strand from the old (Radman et al.,1981; Lu et al.,1983), so we may surmise that the numerous GATC sites may serve to direct the system to the new strand early on in the process of replication.

Another hypothesis that merits consideration is that the delay in methylation which must necessarily occur after replication of oriC due to the large number of GATC sites to be methylated serves as a signal to prevent reinitiation at oriC during that period of the cell cycle when initiation is possible. That this may well be the case is indicated by the results of Messer et al. (1985) who found that the period between successive bursts of reinitiation after return to permissive temperature in a dnaA mutant that had accumulated initiation capacity at nonpermissive temperature could be shortened from 20 minutes to 10 minutes by overexpression of the dam methylase. dam mutants show drastically reduced transformation frequencies with oriC plasmids compared to isogenic dam^{+} strains (Messer et al.,1985; Smith et al.,1985), and unmethylated oriC plasmids are poor substrates for the in vitro initiation system (Hughes et al.,1984; Messer et al.,1985; Smith et al.,1985). However, the effect of dam methylation on in vivo replication

of oriC plasmids and on chromosome replication is still in question (Messer et al.,1985; Smith et al.,1985).

The oriC sequence, along with the identified sites and structural features is shown in Fig. 1.4. Important features of oriC have been defined by comparison of E.coli oriC with sequences of oriC from related bacteria (Zyskind & Smith,1980; Cleary et al.,1982; Zyskind et al.,1981; Zyskind et al.,1983), in vitro site-directed mutagenesis (Oka et al.,1982), in vitro protein binding studies and in vivo and in vitro transcription (Hansen et al.,1981a; Morita et al.,1981; Fuller et al.,1984; Kusano et al.,1984; Lother et al.,1984; Lother et al.,1981; Lother & Messer,1981)

The cloning of replication origins from related bacteria which can function in E.coli and which show homology to the E.coli origin of replication has allowed the identification of a putative consensus sequence (Zyskind et al.,1983). By far the most conspicuous feature of the sequence is its unvarying size: within the minimal origin region, none of the seven bacterial oriC sequences so far determined contain any insertions or deletions, in spite of the fact that a sequence so rich in potential stem-loops provides an excellent substrate for the kind of slippage reactions that are thought to give rise to such mutations (Ripley,1982). The single exception is a 2bp insertion that is balanced by a 2bp deletion only 9bp away in the most distantly related origin sequence, that of Vibrio harveyi. This is consistent with the in vitro mutagenesis results of Oka et al. which show that most insertion or deletion mutations are Ori- even where they occur in non-conserved sequences. The exception to this rule is a 12bp deletion in one of the "spacer" regions (see Fig. 1.4 and text below). The length of this deletion is such that it would only slightly alter the orientation of the surrounding sequences with respect to the direction they faced on the double helix, since 10bp is approximately equivalent to one turn of the double helix. Another smaller deletion of 8bp has an intermediate phenotype while smaller deletions in this region are all Ori-. This suggests that oriC either adopts a particular secondary structure during initiation (for which the various inverted repeats provide a plethora of possibilities), or that it serves as the basis of a large DNA-RNA-protein

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Figure 1.4

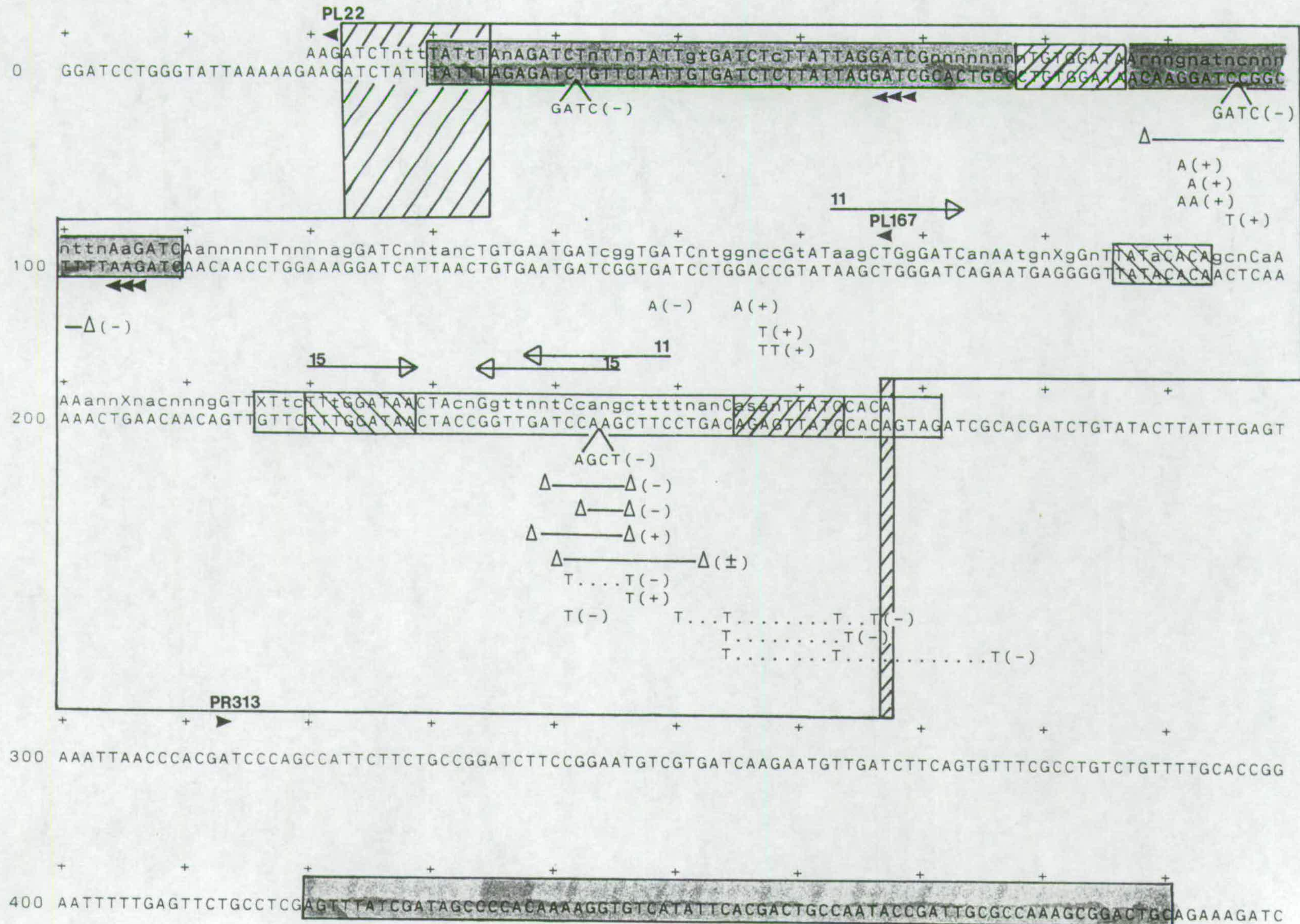


Figure 1.4: The Primary Structure of oriC

The sequence of oriC. The boxed area is the minimal sequence required for autonomous replication (Oka et al.,1980) and the hatched areas indicate the uncertainty of the boundaries of the minimal sequence. The upper sequence is the consensus sequence for the replication origins of *Vibrio natriegens* and five species of the *Enterobacteriaceae*. Completely conserved bases are given in capital letters; lower case letters indicate preferred but not completely conserved bases. X signifies A or G; r signifies C or G; s signifies T or G, and n indicates any base.

Single arrowheads indicate the RNA start points and directions of transcription from the oriC promoters. Hatched boxes indicate the locations of binding sites for the dnaA protein, the two different orientations being indicated by opposite direction of hatching. The open box indicates the gyrase binding site (Lother et al.,1983) and the shaded boxes indicate binding sites for the single-stranded DNA binding protein β' (Lother et al.,1981).

Triple arrowheads below the sequence mark the inception sites (Hirose et al.,1983).

Deletion mutations (Δ----Δ), subsitutions and insertions are indicated with the mutant phenotype marked by them. Inverted repeats nos. 15 and 11 are indicated by open-headed arrows.

complex with a precise stereochemistry. The importance of gyrase for initiation and the large number of suppressors of dnaA mutants certainly lends credence to ideas of a large and elaborate initiation complex with or without an elaborate secondary structure. Hirota et al. (1981) have called this the recognition frame model.

The second conspicuous feature of the consensus sequence is the clustering of conserved bases, a feature which is most noticeable in the outer parts of the sequence. This is consistent with the recognition frame model; the conserved and variable regions being identified as binding sites and spacer regions. By and large, the data obtained from site-directed mutagenesis of oriC support the data from the consensus sequence; Ori+ substitutions being confined to spacer regions and Ori- substitutions being found in conserved regions. The only exception to this rule is an Ori- C to T transition in a spacer region at position 243. Not only is this mutation in a non-conserved sequence, but also the relevant base can actually be deleted from oriC without loss of oriC function (as part of the 12bp Ori+ deletion mentioned above). However, it is noticeable that this deletion replaces C243 with another C residue (and the adjacent A244 with another A), as does the 8bp deletion which gives an intermediate Ori-/Ori+ phenotype. The wild type and mutant sequences in this region are shown below, with the positions of mutations and deletion endpoints underlined:

wild type	TTGATCCAA
Ori- mutant	TTGATCTAA
12bp deletion	TTGATCCAG
8bp deletion	TTGATCCTG

Evidently a C at position 243 is essential to oriC function (the adjacent C242 is an invariant residue). However, in V.harveyi and Erwinia carotovora, the residue at position 243 is A. In the latter cases, a

radically different sequence surrounds the invariant C242 residue while in the other species studied, which are all more closely related to E.coli (Brenner,1984), there is no sequence variation in this region.

The fact that the sequence around position 242 is conserved within bacteria closely related to E.coli, that the C243 is an essential residue in E.coli, and that in more distantly related organisms, this sequence can be greatly different without loss of its ability to replicate in E.coli, suggests that the sequence is involved in the formation of a secondary structure rather than being an essential protein binding site, and that it pairs with another sequence within oriC. Meijer et al.. (1979) have noted two inverted repeats which cover the 243 region. These are repeat no.15 (235-246) which would pair with 219-229 and no.11, (237-250) which pairs with 164-175 (see Fig. 1.4). Examination of the sequence of E.carotovora reveals no corresponding inverted repeats.

This would seem to preclude the need for C243 in the formation of an essential secondary structure. However, I reached this conclusion only by examining the E.carotovora sequence by eye and it is possible that a more systematic search might reveal unnoticed similarities between the potential secondary structures of E.coli and E.carotovora in this region. Alternatively, the sequence differences might indicate significant functional differences between E.carotovora and E.coli oriC in this region. One might for instance suggest that binding of an initiation protein to the 242 region is essential to counteract some inhibitory factor which is not active against the E.carotovora oriC.

Four of the most conspicuously conserved regions are the dnaA binding sites. oriC contains 4 copies of a 9bp sequence which appears to bind the purified dnaA protein (Fuller & Kornberg,1983; Fuller et al.,1984). All sequences so far examined which specifically bind this protein have at least an 8/9 match to this sequence. These include the pSC101 origin of replication, the dnaA promoter region, the "16kd promoter" (the promoter for the gene immediately to the right of oriC at position 765, which directs a transcript which reads into oriC and encodes a 16kd polypeptide (Lother & Messer,1981; von Meyenburg &

Hansen,1980)), the internal repeat of Tn5, and a region close to the replication origin of pBR322 (Fuller and Kornberg, 1983). The first three are known to respond to dnaA activity (Felton & Wright,1979; Atlung et al.,1985 in press; von Meyenburg et al.,1985 in press; Braun et al.,1985; Lother et al.,1985), but what role the dnaA protein may play in the action of Tn5 is unknown. Similarly, it is by no means clear what role, if any, dnaA may play in pBR322 replication. Although pBR322 is unable to replicate in dnaAts strains at non-permissive temperature, integrative suppression by F or R1, but not the mere presence of the plasmid, restores the cell's ability to replicate pBR322 (Frey & et al.,1979); so it would appear that pBR322 replication is dependent on chromosome replication, rather than the dnaA product. However, it should be noted that a search through a sequence database (J.H.Pringle, manuscript in prep.) revealed that sequences similar to the dnaA binding site are quite common in the E.coli genome. This may indicate that the the dnaA product is involved in a wide spectrum of genetic control processes, though it may more likely be a reflection of the similarity of the dnaA binding site to other protein binding sequences (Gicquel-Sanzey & Cossart,1982). It is therefore not at present clear what constitutes an authentic dnaA binding site. Binding of dnaA protein to oriC is significantly enhanced by supercoiling (Fuller & Kornberg,1983).

dnaA binding sites occur in both orientations on oriC, centred at positions 83, 190, 225 and 264; the latter two are close together and in opposite orientations. Binding to these sites may be cooperative, since cleavage of oriC at the HindIII target between them causes a marked reduction in binding efficiency (Fuller & Kornberg,1983). I have mentioned above that a 12bp deletion in this region has an Ori+ phenotype, though smaller deletions are Ori-. It might be suggested that the angle between opposite-facing dnaA binding sites is an important structural feature of this sequence.

This region also contains a site for the binding of DNA gyrase (Lother et al.,1984). It would be interesting to know whether the two molecules cooperate or compete in binding to this region. Although this gyrase binding site has a strong affinity for gyrase, nalidixic acid is

ineffective in inducing this enzyme to cut at this site.

There are several promoters within the oriC region, though none of them has been fully investigated both in vivo and in vitro. Two leftward promoters start transcription near position 167 (Lother et al.,1981) and between positions 22 and -43.The latter has also been identified as an in vivo promoter (Hansen et al.,1981) but the former has only been identified in vitro, possibly due to termination at oriC. I shall refer to these as ori_{PL167} and ori_{PL22}.

Three dnaA binding sites are associated with ori_{PL167}: one, at position 225, occurs 58bp upstream from the ori_{PL167} RNA start site; a second, at position 190, overlaps the Pribnow box (which is an unusually long distance from the RNA start site); and the third occurs 90bp downstream at position 83. Their positions, and orientations are given below (orientation A is the orientation of the dnaA binding site upstream of the dnaA2p promoter that appears to modulate the expression of the dnaA gene):

-50	orientation B
-15	orientation B
+90	orientation A

Hansen et al. (1981) have identified a DNA fragment from position 92 to 244 which contains an in vivo transcriptional terminator, although the in vitro experiments of Lother et al. (1981) did not detect a terminator. It seems possible that either initiation or termination at oriC is modulated by the dnaA protein.

To the right of ori_{PL167} there is a rightward promoter, ori_{PR313}, transcription from which starts at position 313. This promoter lies outside the boundaries of the minimal origin, but it has extensive sequence homology to ori_{PL167} (Lother & Messer,1981) and, like ori_{PL167}, it has a dnaA binding site at position -50 in orientation B.

As I have mentioned above, the presence of a dnaA binding site upstream of the major promoter for dnaA appears to permit regulation of the dnaA gene by its own product. Furthermore, the 16kd promoter is considered to owe its positive effect on oriC initiation (von Meyenburg et al.,1985 in press) to the presence of an upstream dnaA binding site. Thus it seems natural to suggest that the dnaA binding sites 50bp upstream of ori_{PL167} and ori_{PR313} indicate that transcription from these promoters is also influenced by the dnaA product. It is interesting to note that the activity of an in vivo promoter located near ori_{PR313} is destroyed by cleavage at the HindIII site at position 246 (Morita et al.,1981), between the -50 regions of ori_{PL167} and ori_{PR313}, a result which would suggest that the two promoters or their associated dnaA binding sites act cooperatively. This recalls the results of Fuller and Kornberg (1983), which showed that cleavage of oriC at the same HindIII site reduces binding of the dnaA protein in vitro. An alternative explanation would be that the dnaA binding sites are situated at position -90 relative to their respective promoters so that the dnaA binding site modulating ori_{PR313} is the site nearer to ori_{PL167} and vice versa, and that these sites are needed for promoter activity.

ori_{PL167} and ori_{PR313} show a remarkable symmetry of location as evidenced by the fact that the dnaA site which is -50 to ori_{PL167} is -90 to ori_{PR313} and vice versa. Lother et al. (1981) have pointed out that this symmetry is more extended since there is also a symmetrical array of inverted repeats surrounding these promoters.

Drawing on this, the idea of divergent transcription from ori_{PL167} and ori_{PR313} controlled by dnaA and giving rise to symmetrical RNA primers, which are subsequently extended during initiation and give rise to bidirectional replication, seems very persuasive. In vivo inception sites where RNA to DNA transitions occur have been identified at positions 71 and 108 on the strand extending leftward (Hirose et al.,1983), presumably from ori_{PL167}, though this remains conjecture. However, no corresponding sites were found to the right of oriC for the ori_{PR313} transcript. Moreover, ori_{PR313} and its associated inverted repeats can be completely deleted without any obvious effect on oriC plasmid

replication (Oka et al.,1980) though it remains a matter of debate whether bidirectional replication can still occur in this case.

There is no obvious correspondence between the positions of the inception sites and the positions of potential stem-loops or dnaA binding sites However, it is noticeable that both inception sites occur at GATC (dam-methylation sites) in conserved sequences.

The in vivo results of Morita et al. (1981) indicating that the activity of ori_{PR313} is eliminated by cleavage of the HindIII site at 244bp have already been mentioned. Their data also suggest that there is a terminator for leftward transcription in the oriC region. The presence of this terminator may explain the absence of any detectable promoter activity in the fragment containing ori_{PL167} in their experiments.

As yet there have been no reports of characterisation of in vivo transcripts from oriC.

The evidence for transcription in the oriC region thus remains tantalisingly incomplete. A number of major questions remain to be answered :

1) Is ori_{PL167} an in vivo promoter?

2) Is it, like ori_{PR313}, inactivated by cleavage at the HIII site at position 244?

3) Does dnaA modulate the activity of these promoters?

4) Where is the in vivo oriC terminator, and is it influenced by the product of the dnaA gene?.

Outside the minimal origin the promoters read mostly outwards and are apparently simply the promoters for structural genes. ori_{PL22} is the promoter for the gidA gene (Hansen et al.,1981). A further leftward

promoter at position 1326 is the promoter for a 17kd polypeptide of unknown function, while to the right of this is the promoter for the asnA gene which reads rightward from position 1336 (and therefore away from oriC) (Lother et al.,1981). Whether any of these promoters has a role in the control of initiation is unclear, though the position of the gidA promoter and the phenotype of gidA mutants - glucose inhibits division - (von Meyenburg & Hansen,1980) is very suggestive.

The other leftward promoter, at position 765, is more relevant. Transcription from this promoter reading towards oriC increases the copy number of oriC plasmids twofold, irrespective of whether the 16kd protein which it encodes is expressed (von Meyenburg et al.,1985 in press). There is some reason to believe that this promoter is involved in regulation of initiation, since if the transcript is interrupted by a Tn5 insertion in the 16kd gene, cellular DNA content at low growth rates is abnormally high. Also, if a plasmid able to produce all or part of the 16kd transcript is supplied in trans, cellular DNA content in such strains is abnormally low at slow growth rates (von Meyenburg et al.,1985 in press). The 16kd promoter shares the feature of an upstream dnaA binding site with that of the dnaA gene, ori_{PL167} and ori_{PR313}. The importance of the dnaA binding site is clear since the trp and lac promoters are totally ineffective in raising the copy number of oriC plasmids, but the dnaA promoter is as effective as the 16kd promoter (von Meyenburg et al.,1985 in press). The orientation of the upstream dnaA binding site is the same as that for ori_{PL167} and ori_{PR313}, but opposite to that for dnaA2p (Hansen et al.,1982; Atlung et al.,1985 in press; Braun et al.,1985), so seemingly the orientation of the dnaA binding site is not important, at least for copy number effects. It is also worthy of note that the trp promoter, although it has no effect on the copy number of oriC plasmids (von Meyenburg et al.,1985 in press), does have a consensus dnaA binding site upstream (J.H. Pringle pers. comm.). In this case, the site is 100bp upstream of the RNA start point, some 40 to 50 bases further away than those associated with the dnaA, oriC and 16kd promoters. It would be interesting to know if the effect of dnaA on trp attenuation described by Atlung & Hansen (1983) is influenced by the presence of this site.

The association between oriC promoters and dnaA binding sites suggests interaction between RNA polymerase and the dnaA protein in the transcription step in initiation. Furthermore, the enhanced termination at the trp attenuator which the dnaA product mediates, the fact that some dnaA-suppressing alleles of rpoB also enhance trp attenuation (Schaus et al.,1981b; Atlung & Hansen,1983) and the presence of a terminator in the minimal oriC sequence, all suggest that termination might be dependent on the dnaA product. This appears to be the case for in vitro transcription from the 16kd promoter (Lother et al.,1985).

These latter results prompt a reinterpretation of some of the results of Zyskind et al. (1977).

If E.coli cells are treated with rifampicin DNA synthesis slowly comes to a halt. Ongoing rounds of replication are completed, but further initiation of DNA replication is inhibited so that further synthesis is no longer possible. That this is not due simply to the concomitant inhibition of protein synthesis (which has exactly the same effect) can be seen if a dnaA mutant is grown at nonpermissive temperature for a generation or more. During this period it acquires initiation potential: that is it is able to initiate DNA synthesis in the absence of protein synthesis on return to permissive temperature.

If rifampicin treatment is given before return to permissive temperature, DNA synthesis is not resumed.

This has been taken to imply that RNA synthesis is a necessary step which must occur before the action of gpdnaA. However, if streptolydigin (an inhibitor of RNA chain elongation) is used in place of rifampicin, it does not inhibit initiation once initiation capacity has accumulated. This would seem to conflict with the results obtained

with rifampicin.

However, the effect of rifampicin may be a little less simple than it initially seems. The primary effect of rifampicin is on the initiation of transcription and it exerts this effect within seconds of exposure to the cell (Tocchini-Valentini _et_ _al_.,1968). Yet a full inhibition of reinitiation in a _dnaA_ mutant does not occur until 10' after exposure to rifampicin. Thus, unless the transcript is very long, a requirement for ongoing transcription through _oriC_ seems to be ruled out.

Rifampicin, however, has a secondary effect, which is to inhibit the termination of transcription and therefore allow increased readthrough of terminators (Howe _et_ _al_.,1982; Newman _et_ _al_.,1982). The delayed effect of rifampicin could then be explained if, for the initiation of replication, it was necessary for a transcript to be terminated in the _oriC_ region; that rifampicin allowed transcripts paused, but not terminated, at _oriC_ to read through the _oriC_ terminator; and that this effect, unlike that on initiation of transcription, were not immediate.

I would like, therefore, to suggest the following model for _dnaA_ action in initiation of replication:

1) RNA polymerase binds to the _oriC_ promoter and transcribes into _oriC_ until it reaches the terminator, where it pauses.

2) _dnaA_ protein binds to its binding site near the 5' end of the new transcript and travels along this, eventually displacing RNA polymerase and leaving the 3' end free for extension by DNA polymerase. Rifampicin would interfere with this by preventing pausing at the _oriC_ terminator.

Drawing on the known timing of _dnaP_ and _dnaC_ action relative to the rifampicin-sensitive step (Zyskind _et_ _al_.,1977; Wada & Yura.,1974), the apparent requirement for concomitant _dnaB_ and RNA polymerase action, and the known interaction between _dnaC_ and _dnaB_ proteins, the plan of

initiation shown in Fig 1.5 might be proposed.

Horiuchi et al. (1984) have suggested that dnaA serves to protect the oriC transcript from degradation by RNaseH; but, if this were the case, some different explanation would be required to account for the observation that initiation of replication after return to permissive temperature in a dnaAts strain is sensitive to rifampicin but not to streptolydigin: one would expect that all transcripts reading into oriC would be degraded under dnaA- conditions. One might, however, suggest that RNaseH itself is streptolydigin sensitive, and this might also explain why low levels of this drug induce stable DNA replication (Kogoma & Lark,1975).

Beyond the Minimal Origin - Are there Important Control Sequences Left and Right of oriC?

The symmetry of oriC is striking. The sequences around the two promoters ori_{PL167} and ori_{PR313} are closely similar and some 100bp downstream from each is a binding site for the single strand DNA binding protein β', one on each strand (Lother et al.,1981; Jacq et al.,1980)

Yet this structure can be disrupted and oriC still directs replication (Oka et al.,1980). Is the full symmetrical structure required to render replication bidirectional?

Attempts to answer this question in vivo have been made using pOC24 and pCM959 (Meijer & Messer,1980) and in vitro using M13GoriC26 and pSY317(Kaguni et al.,1982). These substrates are perhaps rather ill-chosen to test this hypothesis, since all carry 2 oriC promoters though in the case of pOC24 and M13GoriC26 the rightward promoter reads directly into non-oriC DNA and the symmetrical structures to the right are either removed (pOC24) or separated from oriC by a gap of approximately 14kb (M13GoriC26)

Figure 1.5

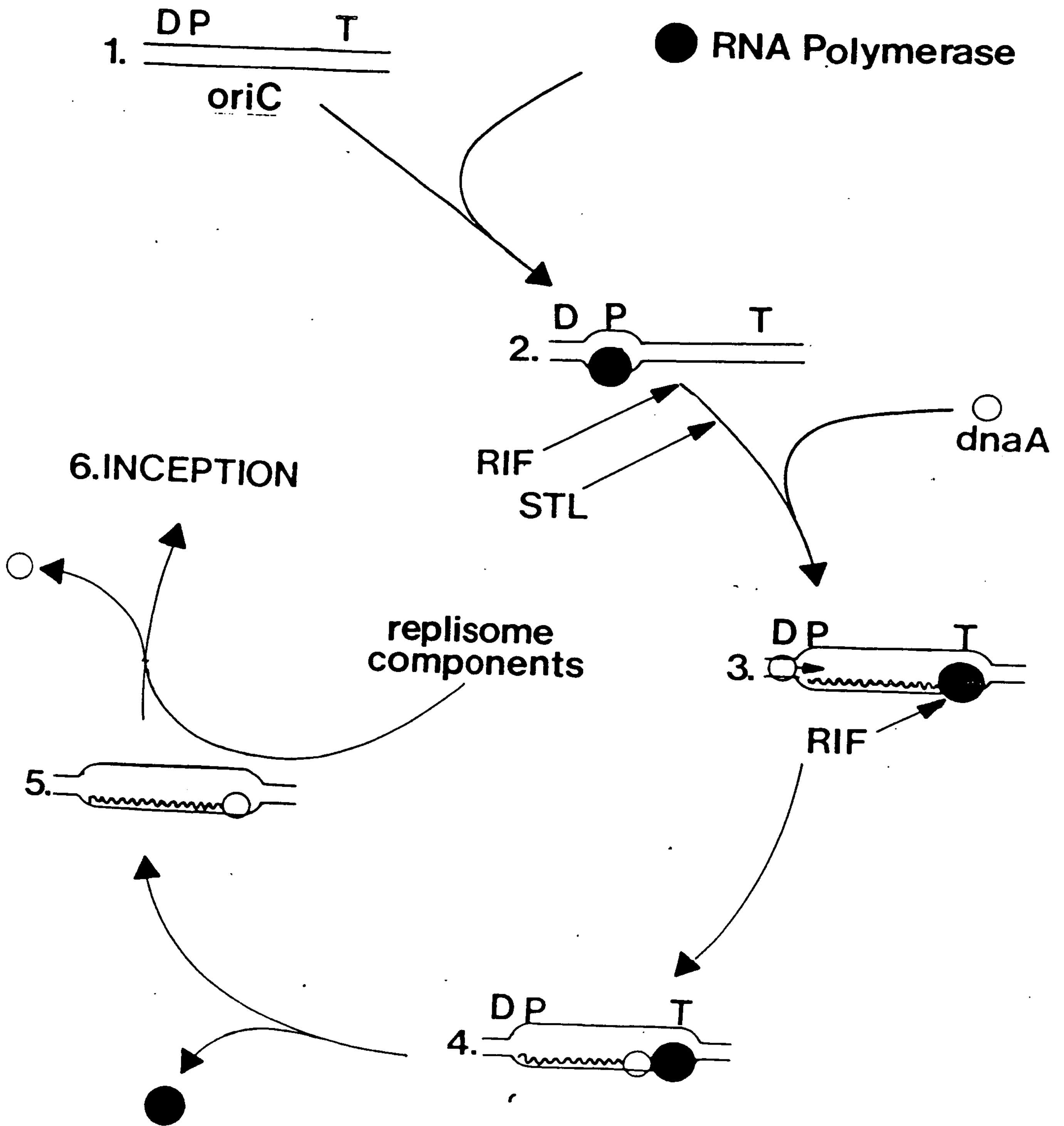


Figure 1.5: A scheme for the action of RNA polymerase and dnaA protein in initiation of replication at oriC

1) oriC DNA: The positions of a dnaA binding site (D), a promoter (P) and a transcriptional terminator (T) are indicated

2) RNA polymerase binds at oriC

3) RNA polymerase transcribes oriC up to the terminator and pauses. Rifampicin (RIF) prevents initiation and streptolydigin (STL) prevents elongation of this transcript. dnaA protein binds to the dnaA binding site upstream of the 5' end of the transcript and moves along one of the 3 nucleic acid strands towards the RNA polymerase molecule.

Rifampicin inhibits by causing RNA polymerase to read through the terminator.

4 + 5) dnaA protein interacts with RNA polymerase completing termination of the paused transcript and releasing RNA polymerase.

6) Inception: Components of the replisome assemble at the 5' end of the RNA primer. dnaA protein is released. DNA polymerase III extends the RNA primer. dnaB and dnaC proteins direct the assembly of the primosome and lagging strand synthesis is initiated.

The in vitro studies, which used dideoxy dNTPs to terminate DNA synthesis (thus giving large yields of replicative intermediates), showed that bidirectional replication was possible from both the substrates they used: the in vivo studies on the other hand were taken to mean that pOC24 directed predominantly unidirectional replication. This relied on the isolation of replicative intermediates by a method that on the whole furnishes depressingly low yields of the desired products. This is reflected in the small number of pOC24 intermediates examined. Furthermore, orientation of the molecules is a difficulty in both studies. Re-examination of the in vivo data reveals that molecules that are well replicated (ie where >3kb of DNA has been replicated) are mostly bidirectional, whereas molecules whose replication has proceeded only a small distance seem generally more likely to be unidirectional.

In fact, this is one area of study where research is hampered, rather than aided by the small size of oriC plasmids. A sceptic might well argue that the evidence for unidirectional replication in a small oriC plasmid whose right hand oriC sequences had been deleted could merely mean that the rightward phase of replication is initiated after the leftward phase, and that in small plasmids replication is often complete before the rightward phase can commence. Two solutions to this difficulty are possible: one is to use the chromosome itself by substituting mutant oriC sequences (with rightward and leftward sequences deleted) for the complete origin and applying any of the well tried methods for marker frequency analysis (such as that of DeMassey et al.,1984) to test for bidirectional replication. The alternative would be to insert appropriate fragments into a large vector molecule of 50 -100kb, where the possibility of unidirectional replication arising merely from lag would be effectively eliminated and where the yield of replicative intermediates would be greatly enhanced due to the greater amount of time required to replicate the molecule.

For the time being, the evidence seems to favour the possibility of bidirectional replication from all oriC derivatives tested, which supports the notion that while leftward replication is RNA-primed, rightward

replication is primed by extension of lagging strand synthesis from the the newly initiated leftward replication fork. However, the smallest oriC fragment capable of supporting replication has yet to be tested for its ability to support bidirectional replication, so for the moment, in the face of somewhat ambiguous data and the lack of work on a true minimal origin the question of what sequences are really required for bidirectional replication remains quite open.

One question that arises from consideration of the conspicuous symmetry and the lack of any convincing evidence that all this symmetry is needed for oriC function is "does E.coli have a double origin?" i.e. is the region to the right of the centre of symmetry able to direct DNA synthesis just as well as that to the left? The data of Oka et al. (1980) indicate that this is not the case since deletions leaving the right hand region of oriC intact, but removing only very small portions of the lefthand region within the boundary of the minimum sequence at 23 - 35 bp abolish oriC function. This suggestion does however offer an evolutionary account of the symmetry, that is that oriC evolved from a pair of back to back unidirectional origins one of which subsequently acquired the capacity for bidirectional replication by an asymmetrical process.

Copy Number and Incompatibility

When F-primes (Masters,1975; Hiraga,1976;), λasn transducing phage (Miki et al.,1978; von Meyenburg et al.,1978a) and oriC plasmids carrying the E.coli origin of replication (Yasuda and Hirota, 1977; Messer et al.,1978) became available, it rapidly became apparent that when maintained as plasmids, these episomes were unstable and deleterious to cell growth (Masters,1975; von Meyenburg et al.,1977; von Meyenburg et al,1978b)

The ability to replicate from oriC was found to be separable from the deleterious effects on cell growth, which mapped to a region to the left of oriC called het (von Meyenburg et al.,1977).

The effect of *het* was very much what might be expected if it caused incompatibility between the plasmid and the chromosome: that is, cells carrying *het* plasmids had a low DNA content, tended to form long cells in addition to normal sized ones, and were slow growing (von Meyenburg *et al*.,1977; Wada *et al*.,1977; Masters,1975).

It has been found, however, that overexpression of the *atp* operon, whose promoter region overlaps *het*, also causes aberrant cell morphology due to crowding of the cell membrane with components of the membrane bound ATP synthetase (von Meyenburg *et al*., 1984).

Ogura *et al*. (1980), by selection for increased antibiotic resistance, isolated mutants of *oriC* plasmids, which they called *cop*, which raised the copy number of *oriC* plasmids. These mapped to the *het*/*atp* region. Hansen *et al*. (1981b) isolated similar mutants in λ*asn* phages, with a similar genetic locus, which they showed to be defective in expression of the *atp* operon.

Two explanations have been advanced to account for mutants of this kind. Hansen *et al*. consider that the high copy number observed for their *atp* mutants is due to the relief of deleterious cell growth, which otherwise forces copy number down (see below). Ogura *et al*. consider their *cop* mutants lack a control locus coding for the production or response to a repressor of initiation, and/or involved in partitioning of the chromosome, and thus have a high copy number and do not express incompatibility with the chromosome. Were this the case, one would expect the mutants to affect the copy number and/or stability of the chromosome when transferred to it. This has been done with the *atp* mutants of Hansen *et al*. (1981b) and they seem to have a normal chromosomal copy number (Hansen *et al*.,1981b). Seemingly, the relief of the *het* phenotype associated with *cop* and *atp* mutants is simply due to the relief of the pressure of excess *atp* operon synthesis.

oriC plasmids that lack *het* are much less deleterious to cell growth (von Meyenburg *et al*.,1977 & 1978b), less unstable, and have higher copy numbers; all of which would be predicted if *het* were an incompatibility

region. However, measurement of the copy number of such plasmids is somewhat fraught with difficulties.

In the case of plasmids such as colE1 or the incFII group, negative control by a small RNA molecule ensures that all cells which inherit the plasmid have the same number of plasmid copies (Nordstrom,1983).

However, in the case of oriC plasmids, we have no a priori reason for believing that such a copy number control system exists. If, like the chromosome itself, oriC plasmids initiate replication only once per cell cycle, and if the plasmids partition randomly (as their instability suggests), selection against plasmid-free cells will tend to increase the overall copy number of plasmids in the cell population, since the plasmid population increases as the Nth power of 2 (where N is the number of cell generations) while the number of plasmid-carrying cells increases less rapidly due to the effects of plasmid instability. This would continue until limitations on some initiation or replication component, or the deleterious effects of plasmid-encoded genes, balanced copy number increase by loss of plasmid-free cells, and the result would be a continuous distribution of copy number between 0 and the upper limit. The mathematics of plasmid loss under such circumstances have never been worked out; but it is clear, even without attempts at mathematical calculation, that in such a distribution, conditions of selection (for instance, the minimum copy number compatible with resistance to a given antibiotic level) would affect the measured copy number.

In addition to problems of this sort, the apparent rate of plasmid segregation under non-selective conditions can be augmented by several factors: plasmid-free cells may grow faster (as is the case with het plasmids); stability (and therefore observed copy number) is strain-dependent (Yamaguchi et al.,1982); association of oriC plasmids to different extents with the cell membrane, as would be expected from the fact that different oriC plasmids contain different numbers of sites for the membrane protein β' (Lother et al.,1981), may differentially affect recovery of plasmid DNA; and het plasmids can integrate into the

chromosome by an efficient recA-independent process (Masters et al.,1975). All of these influences are likely to affect the measurement of copy number, and it is in the light of this that all discussion of unstable oriC plasmids should be examined.

In addition to the het region, the region to the right of oriC also appears to be relevant to copy number. The presence of the promoter of the 16kd gene seems to increase copy number and stability (Stuitje & Meijer,1983), although by a different method, in a different strain background, Yamaguchi et al. (1983) found a reduction of copy number due to the region containing this promoter.

A third class of copy number mutations, which has unfortunately received little attention, is carried by the pseudovirulent mutants of λoriC (Soll,1980). These phages, due to a mutation in oriC, are able to form plaques on a homoimmune λ lysogen. Presumably, this is due to their being copy number mutants of oriC, which allows them to attain copy numbers high enough to titrate out the λ repressor and thus induce the lytic cycle. Since in such a system, the effects of oriC on cell growth will have so much less effect on "copy number", these mutants may prove useful as sources of copy number mutations that would be lethal on a plasmid which must be maintained in a living cell. Should mutants in the cop region appear among these pseudovirulent mutations, it would be strong evidence for the involvement of this region in copy number control rather than merely the expression of potentially deleterious genes. Sadly, the mutations have not been accurately mapped.

Incompatibility

oriC plasmids are incompatible with each other; that is, when two oriC plasmids are maintained in the same cell, selection for one of the plasmids results in a loss of the other faster than would be expected if the plasmid were being maintained alone in the cells. However not all oriC plasmids show this effect.

Yamaguchi et al. (1982) have shown that pBR322 derivatives carrying either of two segments of the oriC region increase the instability of another resident oriC plasmid which can replicate only via oriC. These segments are in the region between positions 1 and 276, which contains the minimal origin sequence, and the region between positions 288 and 1050, which carries the 16kd gene. Both these regions are transcribed in vitro, and both could produce a polypeptide, although in the case of the 1-276 fragment, this would be only 27 residues long. Both also carry dnaA binding sites and sites for binding to the membrane protein β' (Lother et al.,1981). It seems likely that these inc regions either express some repressor of initiation, or compete for some limiting initiation or replication factor, so that the copy number of the oriC-dependent plasmid is reduced. It is interesting to note that, in common with binding of the dnaA protein (Fuller and Kornberg,1983) and activity of the in vivo rightward oriC promoter (Morita et al.,1981), expression of incompatibility by the incA region (which overlaps the minimal origin) is eliminated by cleavage at the HindIII site at position 265.

Similar studies by Stuitje and Meijer (1983) demonstrated the existence of three incompatibility regions: incA, in the same region as that found by Yamaguchi et al. (1982) in the minimal origin region; incB, which mapped very close to the promoter for the 16kd gene; and incC, which appeared to correspond to the coding sequence of the 17kd gene to the right of the 16kd gene. Unlike Yamaguchi et al. they found that none of these regions was able to cause incompatibility on its own, but required the presence of one of the other inc regions. The results of these various combinations are shown in Table 1.6.

The differences between the results of Yamaguchi et al. and those of Stuitje & Meijer may be due to the fact that the oriC plasmids against which incompatibility was expressed in the experiments of Yamaguchi et al. lacked the incC locus, while in the experiments of Stuitje & Meijer, the plasmids were incC+.

Table 1.6 Strength of incompatibility shown by various combinations of inc loci

inc loci	incompatibility
incA	-
incB	-
incC	-
incA, *incB*	++
incA, *incC*	+
incB, *incC*	+
incA, *incB*, *incC*	++

At least three components seem therefore to be involved in oriC incompatibility: the origin of replication itself (incA), the promoter for the 16kd gene (but not its coding sequence, most of which can be removed without effect on incompatibility), and the gene for the 17kd polypeptide. That it is the products of these incB and incC genes that cause incompatibility is indicated by the effect of transcription into the incB-incC region against the normal direction of transcription, which abolishes expression of incompatibility. That transcription reading into oriC from the 16kd promoter is not responsible for incA action is suggested by the finding that the whole of pBR322 can be inserted between them without affecting incompatibility.

Since none of these plasmids shows incompatibility with the chromosome, as indicated by the normal growth rates of strains carrying them, we may ask what bearing these results have on the control of chromosome replication. One possibility is that oriC, under the conditions used here, is controlled by a different system, and the incB, incC system is not used unless the former system becomes inoperative for some reason. In this case, a survey of the effects of incA, incB, incC plasmids under various conditions of growth might prove instructive.

Other Replication Origins

Besides oriC, the E.coli chromosome carries at least four other replication origins.

oriJ

OriJ is the replication origin of the defective lambdoid prophage rac (Diaz et al.,1979). This prophage does not produce virions nor does it kill its host, but its induction can be observed as a transient suppression of the recombination deficiency of recBC strains due to the derepression of the prophage encoded exonuclease VIII, the sbcA or recE

product, which substitutes for the recBC-encoded exonuclease V (Low,1973; Kaiser & Murray,1979a). This phenotype is seen when the prophage is transferred to a Rac- background, such as AB1157 or AB2463 (Low,1973). Replication of oriJ is similarly only observed in Rac- strains (Diaz et al.,1979). oriJ plasmids probably closely resemble λdv (Berg,1974; Matsubara,1976; Matsubara,1981) in their behaviour and it is doubtful whether chromosome replication could be driven by oriJ without serious ill effects on the chromosome. Furthermore, induction of rac also causes its excision from the chromosome in addition to replication (Low,1973).

oriK

In rnh strains, constitutive for stable DNA replication, four (possibly five) origins of replication are recognised. These are collectively known as oriK (DeMassey et al.,1984). They are located at 45', 95', and close to the terminus, where two very closely spaced origins occur, at 30.5 and 32.5 minutes. The former of these two might correspond to oriJ. A possible fifth origin at 20' may also exist. Replication from these origins probably results from the failure of the defective RNaseH to degrade their RNA primer molecules.

Attempts to transform rnh-,dnaA- strains with oriC plasmids were not successful, indicating that stable DNA replication cannot recognise oriC. It is not yet clear whether the origins identified by DeMassey et al. are the same as those recognised in inducible stable DNA replication and in SdrT- strains.

The Biochemistry of Initiation

One of the most exciting recent developments in the field of DNA replication is the construction of an in vitro initiation system specific for oriC (Fuller et al.,1981). Replication of phage and plasmids in vitro has been under study for some years (Tomizawa et al.,1981; Kolter et al.,1978; Diaz et al.,1981; Wickner,1981; Kornberg,1978; Kornberg,1982;

Eisenberg,1976) but crude E.coli extracts had not been found to be active with oriC (Fuller et al.,1981) although a cellophane membrane/lysed cells system could be used (Schaller et al.,1972; Nusslein-Crystalla & Scheefers-Borchel,1979).

The lack of activity of crude lysates appears to be due to the presence of inhibitory factors, since activity can be achieved by rather precise fractionation of lysates with ammonium sulphate (Fuller et al.,1981).

The system has a very marked resemblance to the in vivo replication system in the following characteristics:

1) It is specific for oriC: other double stranded templates are either inactive or much less active; ColE1 being the most active alternative template tested. The dnaA-dependent plasmid pSC101 is inactive as a template, presumably due to dependence on a plasmid-encoded replication protein (Fuller et al.,1981. Linder & Churchward,1985).

2) dnaA, dnaB and dnaC proteins are required (Kaguni & Kornberg,1984; Fuller & Kornberg,1983; Fuller et al.,1981).

3) The reaction is inhibited by rifampicin only when this is added at the beginning of the reaction (Kaguni & Kornberg,1984).

4) Nalidixic acid, and to a lesser extent novobiocin, inhibit the reaction. (Fuller & Kornberg,1981).

5) In the absence of RNaseH, the system loses its specificity for oriC (Ogawa et al.,1984).

6) Bidirectional replication can occur (Kaguni et al.,1982).

Other features of the system are:

a) Dependence on supercoiling: relaxed or linearised *oriC* plasmids are far less active as substrates than supercoiled ones, and the specificity of the system is less; linear ΦX174 is almost as good a substrate as linearised *oriC* (Fuller & Kornberg,1983). The role of gyrase in the *in vitro* system is not clear, since the primary effect of nalidixic acid may be to cause DNA damage by gyrase. Extracts of temperature-sensitive gyrase mutants have yet to be tested for their activity; thus the active involvement of gyrase in initiation remains unproven *in vitro* as *in vivo*.

Topoisomerase I both stimulates the activity of the system and increases its specificity for *oriC* (Kaguni & Kornberg,1984). *In vivo*, this enzyme serves to counteract excessive supercoiling by gyrase by relaxing supercoils (Pruss *et al*.,1982) and, as has been mentioned above, mutants in *topA* suppress *dnaA* (Louarn *et al*.,1984). Thus, the *in vivo* and *in vitro* effects of topoisomerase I are consistent.

b) The *ssb* protein is required. This protein is an important factor in a number of *in vitro* reactions including:

1) RNA polymerase priming of M13 single strand DNA replication at a stem loop that marks the origin of replication and has no obvious resemblance to a promoter (Kaguni & Kornberg,1982).

2) Assembly of the primosome (consisting of proteins n, n', n'', i, *dnaB*, *dnaC* ,and *dnaG* primase) that primes lagging strand synthesis in ΦX174 SS to RF conversion, *ssb* protein being required for the binding of protein n' to DNA (Shlomai & Kornberg,1980).

E.coli chromosome replication is dependent on *ssb* protein *in vivo*, as *ssb* mutants are defective in DNA replication (Bouche *et al*.,1975).

One of the unanswered questions about the *in vitro* initiation system is the nature of its product, although answers to this question are beginning to emerge. Initially, the *in vitro* reaction appeared to depend on a hydrophilic polymer such as PVA to provide "macromolecular crowding" and the use of this resulted in highly catenated DNA being formed (Low *et al.*,1984), which may explain why the reaction ceased after 30 minutes (Fuller *et al.*,1981). Excess gyrase α subunit has now been shown to be able to substitute for PVA and the resulting product is uncatenated (Kaguni & Kornberg,1984), although whether this product is a substrate for further initiation has not been reported.

Purification of the essential components of the reaction and its *in vitro* reassembly (Kaguni & Kornberg,1984) throws some light upon the order of events in initiation at *oriC*. RNA polymerase has been shown directly to be necessary for initiation *in vitro*, since its omission from the reaction mix abolished replication. Furthermore, the RNA polymerase step could be shown to precede all other steps in the reaction, since RNA polymerase action followed by the addition of all other components of the reaction mix plus rifampicin and streptolydigin was sufficient to give synthesis. This confirms a prediction of the model suggested above; that productive RNA synthesis should occur in the absence of *dnaA* activity.

It has been mentioned that *in vivo*, *dnaB* protein seems to be essential for the transcription step in initiation, as *dnaB252* mutants cannot initiate unless conditions permissive for RNA synthesis and the mutant gene product prevail simultaneously (Zyskind & Smith,1977). Since the *dnaB* protein is not needed for the *in vitro* transcription step, I am inclined to favour the alternative hypothesis: that *dnaB252* product "poisons" initiation at non-permissive temperature.

The components of the purified system are: DNA polymerase III holoenzyme, primosome components (*dnaG* protein, *dnaB* product, *dnaC* product, proteins n, n', n'', i), *dnaA* protein, single strand binding protein, DNA gyrase β subunit, and the specificity factors topoisomerase I, RNaseH and HU protein (Kaguni & Kornberg,1984). This last factor is a

highly basic protein which selectively enhances replication at oriC (Dixon and Kornberg,1984). Notable for their absence from this list are the products of the dnaI and dnaP genes, though it is still possible that these products are present in sufficient quantities as contaminating activities in the preparations of some other factor or factors, as has been suggested as an explanation for the fact that omission of the gyrase α subunit does not completely abolish activity.

Lastly, the in vitro system is independent of membrane fractions. A role for the cell membrane in the control of initiation and segregation has long been a favoured hypothesis (Jacob et al.,1963) and is supported by some circumstantial evidence (Lark & Lark,1966; Smith & Hanawalt,1967; Fralick & Lark,1973). Covalent binding of the origin of replication to an outer membrane component has been found (Wolf-Watz & Masters,1979) and there are many reports of a selective binding of the origin to membrane fractions (Kusano et al.,1984; Hendrickson et al.,1981; Jacq et al.,1980; Hendrickson et al.,1982; Nagai et al.,1980; Nicolaides & Holland,1978). Yet if the in vitro system is not artefactual and since it depends exclusively on soluble enzymes (Fuller et al.,1981; Kaguni & Kornberg,1984) it seems that membrane binding is not actually required for initiation. It possibly plays a negative role in the reaction or is required for segregation or the orderly folding of the newly replicated chromosome.

Concluding Remarks

After many years of study, the various elements of the initiation of chromosome replication are beginning to draw together so that a coherent whole may soon start to emerge. In the area of the genetics of initiation, this may reflect the increasing saturation of the genetic map so that, for instance, the discovery that the loci dasF (dnaA suppression), sdrA (stable DNA replication), cer (colE1 replication) and rnh (RNaseH) were in the same gene has led to a unification of these four phenotypes (Kogoma et al.,in press) and a consequent increase in our understanding of DNA replication and its control. We may expect this

to happen to an increasing degree. In other areas, *in vivo* studies of the effects of antibiotics and temperature sensitive mutations are becoming explicable in terms of the *in vitro* biochemistry of initiation as has been detailed in the preceding section.

In the near future we may expect to see a biochemical mechanism for initiation *in vitro* emerge. The study of copy number mutants of RNA polymerase (Tanaka *et al.*,1983; Rasmussen *et al.*,1983) and the study of *oriC* transcription promises to clarify the apparent transcriptional control of initiation and the role of the *dnaA* protein. Ultimately (and preferably sooner) we will wish to know what the nature of the *in vivo* *oriC* transcripts are.

Our knowledge of protein binding to *oriC* is advancing rapidly, and it is to be hoped that the "footprinting" techniques will be brought to bear on the precise nature of the interactions, as has been done with the *dnaA* protein (Fuller *et al.*,1984). The significance of association between the cell membrane and *oriC* is one area which is in need of clarification. A conditional mutant in the *oriC*-binding β' protein, for instance, would greatly enhance our understanding of the significance of *oriC*/membrane interactions, as would a study of the much neglected *dnaP* gene, whose phenotypes are so suggestive of membrane alteration (Wada & Yura,1974).

The idea that the replication origin, or the RNA primer that primes initiation, adopts a secondary structure during initiation seems to warrant re-examination. It seems likely that enough information from mutagenesis of *oriC* and comparison of bacterial *oriC* sequences has now accumulated to allow us to eliminate a sufficient number of the bewilderingly numerous possible secondary structures for *oriC*, so that we can begin to make predictions about what structural features (if any) may be important for *oriC* function, in other words, to develop a "consensus structure" of *oriC*. The new and very precise methods of *in vitro* mutagenesis (Messing, 1983) which are becoming available, mean that it should prove possible to test theories about the significance of the binding of initiation proteins to various parts of *oriC*. The many Ori-

mutants isolated by Oka et al (1982) offer an opportunity to look for important interactions between proteins and DNA sequences in oriC by seeking suppressor mutations which alter the binding specificity of initiation proteins and thus restore oriC function. The power of such methods to elucidate protein/DNA interactions is elegantly demonstrated by the work of Ebright et al., (1984)

Suppression analysis may be used to study protein/oriC interactions, nucleic acid interactions within oriC, protein/protein interactions, or to elucidate the genetic control networks that integrate initiation of replication into the cellular physiology of E.coli. The work presented in Chapter 3 strongly suggests a hitherto unsuspected interaction between the dnaA and groE genes.

Objectives of the project

Chapters 3 to 5 describe three sets of experiments united by the common theme of the initiation of DNA replication.

In chapter 5, experiments are described which were aimed at isolating chromosomal origins of replication both from species within the Enterobacteriaceae and from genera more distantly related to E.coli, such as Alcaligenes. This was intended to allow, in addition to sequence comparison, the comparison of the ability of related oriC sequences to disturb cell growth by mechanisms analogous to the Het phenotype (von Meyenburg et al.,1977; Wada et al.,1977; Masters,1975) and to integrate into the chromosome via recA-independent processes (Masters et al.,1978), as well as comparison of the stability of these plasmids.

Difficulties in isolating and characterising authentic oriC plasmids necessitated a more modest approach to sequence comparison, and to this end, the potential of Southern Hybridisation for assessment of sequence similarity was explored. These experiments are described in chapter 4. Probes containing various segments of DNA containing oriC have been employed to locate the most highly conserved parts of the oriC region. Probes from other parts of the E.coli chromosome were

used by way of comparison to identify possible evidence of lateral transfer of oriC sequences between bacterial species.

Since dnaA is a gene believed to encode an initiation protein whose action is specific for oriC (Nishimura et al.,1971; Nishimura et al.,1973; Moody & Runge,1972; Goebel,1973; Goebel,1974; Lindahl et al.,1971; Tresguerres et al.,1975; Chesney & Rothman-Scott,1978) it was considered of interest to investigate the possible co-evolution of oriC and dnaA. A protein and a DNA sequence that interact in an obligatory and sequence-specific fashion are expected (a) to constrain each others' sequence divergence due to the need for change in the interacting sequence of one partner to be compensated by sequence alteration in the other, and (b) to be unable to be separated by lateral transfer, since each version of oriC will require its own (or a very closely related) version of the dnaA product, and lateral transfer should only occur if both components are transferred together. Thus, it should be possible, given the sequence similarity of two oriC sequences, to predict the similarity of the corresponding dnaA sequences. Methods described in Chapter 4 should allow this hypothesis to be tested.

This line of reasoning has to some extent been overtaken by facts. The chromosomal origin of replication of Vibrio harveyi, which is sufficiently diverged from that of E.coli to make the homology of the two undetectable by hybridisation (Zyskind et al.,1983) is able to replicate in E.coli and thus presumably to utilise the E.coli dnaA product. Apparently, the co-evolutionary constraints imposed by oriC and dnaA on one another are fairly lax, and, in the case of the oriC sequence, presumably restricted to the 4 conserved 9bp dnaA binding sites (Fuller et al.,1984).

Attempts to clone the dnaA gene for use as a probe for comparison with oriC resulted in the isolation of a suppressor of dnaA. The characterisation of this suppressor is described in Chapter 3.

The degree of saturation of the E.coli genetic map gave good reason for hoping that this suppressor gene could be equated with one of the

known genes, and thus that a hitherto unknown interaction of dnaA could be identified and new insights into the mechanism of initiation at oriC be gained. For this reason, the mapping of the genetic locus of the suppressor was undertaken and several novel methods of genetic mapping were explored to tackle the problem of mapping using vector markers rather than chromosomal markers. Allele specificity studies were undertaken to narrow down the possible mode of suppression (see pp 29 - 32) and various aspects of the physiology of suppressed strains were examined, in particular growth rates, DNA synthesis and cell division, in an effort to assess the completeness of suppression and its effects on the parameters of the cell cycle.

Chapter 2: Materials and Methods

2.1 Bacterial and Phage Strains

Bacterial strain are listed in table 2.1. Bacteria were maintained on LA plates stored at 4°C for regular use, or in 0.7% nutrient agar stabs at room temperature. For long term storage of unstable strains (strains carrying F-primes, unstable plasmids, mutant strains having a selective disadvantage over revertants, and cold-sensitive strains, early stationary phase cultures grown under selective conditions were harvested by centrifugation and resuspended in 2xLB to which 15% glycerol was added, or alternatively resuspended in 50% glycerol in phosphate buffered saline (Table 2.4) and stored at -20°C.

Special media were used for the maintenance and growth of *Azotobacter vinelandii*, *Rhizobium leguminosarum*, and *Vibrio natriegens*. These strains were maintained on 1.5% agar plates or 0.7 agar stabs of the appropriate growth media (see table 2.3).

Bacteriophage strains are listed in table 2.2. Phage lysates were stored at 4°C either as broth suspensions or as high titre purified suspensions in CsCl in phage buffer.

2.2 Growth Media and buffers.

Growth media and buffers are listed in tables 2.3 and 2.4 respectively. L-broth (LB) and L-agar were used for all bacterial cultivation except where otherwise stated. For work with phage λ, these media were supplemented with 10mM $MgSO_4$ and for P1 with 1mM $CaCl_2$. VB agar supplemented with appropriate carbon sources, vitamins and amino acids was used for the selection of nutritional markers.

Table 2.1a. E.coli K12 strains

strain	description	origin	reference
AB2569	*proA2,hisG4,argH1,metA28, thi-1,lacY1,galK2,xyl-5, mtl-1,supE44?,tsx-29*(T6^R), λ^-	CGSC	
AB259	*Hfr:PO1,thi-1,relA1,*	J.Maule	Jacob & Wollmann, 1956
AJN30	*metB,his,thi, rpsL*(Str^R)	R.S. Hayward	
AM1	*Hfr:PO2A,pfkA1,relA1, fhuA22*(T1^R,Φ80^R,T2^R), *pit-10,spoT1,*	CGSC	Morrissey & Fraenkel,1969
C600	*thr-1,leuB6,thi-1,supE44, lacY1,fhuA21*(T1^R, Φ80^R)	Laboratory stock	Appleyard,1954
C600K-	*thr-1,leuB6,thi-1,supE44, lacY1,galK,fhuA21*(T1^R, Φ80^R)	K.Begg	
χ478	*leu,lac,proC,purE,trp, lys,metE,ara,xyl,mtl, thi*,T1^R, T6^R,*rpsL*(Str^R)	Laboratory stock	
CM5649	*metB1,leu*(Am),*trp*(Am), *lacZ*(Am),*galK*(Am),*galE, sueC,tsx,relA,supD43, rpsL*(Str^R),*sueB,polA21*(Am), *zif-1863::Tn10*	W.Kelley	Kelley & Joyce, 1983
CND19	*thr-1,leuB6,trp-67, his-100,met-99,dnaP18, thiA1,ara-13,lacY1, gal-6,xyl,mtl-2,azi-8, rpsL135*(Str^R),*fhuA2*(T1^R), *supE44*	Laboratory stock	Wada & Yura,1974

Table 2.1a continued

strain	description	origin	reference
CND20,	groF, supF, λ^{s}	Wil Loenen	
CS8/ASP23	fhuA22,ompF627,relA1, spoT1,gltS$_{0}$8,metB1, aspA23,Hfr:PO2A	CGSC	
E177	leuB6,thr-1,thi-1,thyA6, deoC1,lacY,rpsL67(str^{R}), fhuA(T1^{R},ϕ80^{R}),λ^{-}, supE44,dnaA177	Laboratory stock	Wechsler & Gross, 1971
ED2433	Hfr,Δ(gpt-lac)5,thi, met	Laboratory stock	
ED3029	Hfr,Δ(gpt-lac)5,thi	Laboratory stock	
ED3062	Hfr,Δ(gpt-lac)5,thi	Laboratory stock	
ED419	leu,Δ(lac-proB),supE42, dnaA46,Tlr(?)	N.S.Willetts	Bachmann,1972 Caro & Berg,1968 Bird et al.,1976
ED535	Δ(lac)X74,his,lys trp,gal,sup^{o},rpsL(Str^{R}), tsx(T6^{R}),pED936	G Coupland	Brown,1981
ED536	F^{-},Δ(lac)X74,his, lys,trp,gal,sup^{o}, rpsL(Str^{R}),tsx(T6^{R}),pED936	G Coupland	Brown,1981
ED8641	hsdR,hsdM^{+},metB1,gal, Δ(trpB-trpE)9,trpR, lacY1,supE44,recA56	Laboratory stock	
H1175	leu-32,proA35,argF58, purA45,argI60,lacY1,gal-6, malA1(λ^{R}),xyl-7,mtl-2, rpsL125(Str^{R}),tonA48, tsx-70,λ^{-},supE44(?)	CGSC	Glansdorff,1967
J62Tn7	lac-28,his-51,trp-30, proC2,zid-978::Tn7	laboratory stock	

Table 2.1a continued

strain	description	origin	reference
JF279 rec+	*his,argG,leu,metB,ilv, pyrB,rbs,mtl,xyl,gal, lac,malA*,Nal^R,Str^R,Spc^R	Laboratory stock	Masters.1977
JF50	*F$^-$,mel,Δ(lac),gyrA* (Nal^R), *supF*	A.Wright	. . . . .
JG138	*thy,rha,lac,polA1*, Str^R	Laboratory stock	. . . .
JM101	*Δ(lac-pro),supE,thi/ F′traD36,proAB$^+$,lacI^q lacZΔm15*	Laboratory stock	. . . .
JW154, pSP6	*thyA36,deoC2,rha-5,lacZ53, rpsL151*(Str^R),*dnaA508*,pSP6	J.Wechsler	Projan & Wechsler, 1981
JW397	*metE70,thyA36,deoC2, malB45,rha-5,lacZ53, rpsl151*(Str^R),*dnaA5, zib501::Tn10*	J.Wechsler	. . . .
JW398	*metE70,thyA36,deoC2, malB45,rha-5,lacZ53, rpsL151*(Str^R),*dnaA5*	J.Wechsler	. . . .
JW402	*his,trp,Λ(lac)X74,dnaA508, rpsE*(Spc^R),*zib501::Tn10*	J.Wechsler	. . . .
Lin61	Hfr:PO2A,*glpK1,lac-20, relA1,phuA22*(T2^R),*pit-10, spoT1*	CGSC	Hayashi & Lin,1965 Cozzarelli & Lin, 1966
M2508	*Hfr:PO2A,metB1,relA1, melA7,spoT1*	CGSC	. . . . .
M5000	*trpA9825,rpsL196,StrR, glnA2*,λ$^-$,*IN(rrnD-rrnE)1*	CGSC	Mayer et al.,1975

Table 2.1a continued

strain	description	origin	reference
MM303	*thiA1,ilv-192,argH1,metB1, pyrE41,xyl-7,uhp-2 tnaA1,lacY1* or *lacZ4, Δ(trp-tonB),tsx-7*(T6^{r}), *rpsL8,9* or *17*(Str^{r}),(P1)	Laboratory stock	Masters,1977
MM304-2	*hisG1,ilv-192,tnaA1, pyrE41,Δ(trp-tonB),xyl-4, rha,uhp-2,malA*(λ^{R}), *lacY*,Str^{R},T6^{R}	Laboratory stock	Masters,1977
MM311	*thi-1,ilv-192,argH1, metB1,xyl-7,malA1*(λ^{R}), *hisG1,lacY,tnaA1,ara-13 tsx-7*(T6^{r}),*rpsL8,9* or *17*(Str^{r}), *supE44,Δ(trp-tonB),uhp-2, mel-1,pyrE41*,λ^{-},(P1)	Laboratory stock	Masters,1977
MM7	*argG,leu,metB,his,pyrE, ilv,malA*(λ^{R}),*xyl7,mtl*$^{+}$, *lac,gal,uhp*,Nal^{R},Str^{R},	Laboratory stock	
ND11	*metB1,trpR,hsdR,hsdM*$^{+}$, *sup*^{o},*asnB54::Tn5,asnA*$^{+}$, *Δ(trpB-trpE)9,gal, lacY1*	ND10 (ED8641, *recA+*) x P1/R2D2 to *asnB::Tn5, supD+*	
ND13	*metB1,trpR,hsdR,hsdM*$^{+}$, *sup*^{o},*asnB54::Tn5,asnA*$^{+}$, *polA21*(am),*gal,lacY1, Δ(trpB-trpE)9 Δ(metE)113*	ND12 (ND11 *polA21, zif-1863::Tn10* /P1 on CM5649) to TetS,by Quinaldic acid curing	

Table 2.1a continued

strain	description	origin	reference
ND130	*metB1,trpR,hsdR,hsdM*$^{+}$, *sup*^{o},*asnB54::Tn5,asnA*$^{+}$, *polA21*(am),*gal,lacY1*, *Δ(trpB-trpE)9* *Λ(metE)13,pHR3*	ND13 x pHR3	. . . .
PC1	*leuB6,thyA47,deoC3,dnaC1*, *rpsL153*(StrR),λ^{-}	Laboratory stock	. . . .
PC2	*leuB6,thyA47,dnaC2,deoC3*, *rpsL153*(StrR),λ^{-},*dnaT2*,	M.Hepburn	. . . .
R2D2	*AsnA31,asnB54::Tn5*(Kan^{R}), *Λ(lac),thi,ara,gyrA* (Nal^{R})	J.Felton	. . . .
RS162	*thr-1,leuB6,thyA6*, *zjb540::Tn10,dnaB252*, *deoC1,lacY1,rpsL67*(Str^{R}), *tonA21*,λ^{-},*supE44*	Laboratory stock	. . . .
RSH431	*metB,his,thi,rpsL*(Str^{r}), λAJN172(RifR)	R.S.Hayward	. . . .
SM32	*his,pyrD,Λ(lon)1000*, *sulA,gal,rpsl*(Str^{R})	Frances Pace	. . . .
W3110	IN*(rrnD-rrnE)1*	Laboratory stock	. . . .
WM1026	*lac,supD,thi,trp*, *dnaA46*	W. Messer	. . . .
WM1029	*lac,supD,thi,trp*, *dnaA167*	W. Messer	. . . .
WM1032	*lac,supD,thi,trp*, *dnaA508*	W. Messer	. . . .
WM1152	*lacI*^{q},λ^{-},*dnaA204*,	W. Messer	. . . .

Table 2.1a continued

Strain	Description	Origin	Reference
E.coli B/r			
JW393	*leu-19*, *pro-19*, *trp-25*, *his-47*, *thyA59*, *arg-28*, *ilv*, *deoB23*, *lac-11*, *gal-11*, Spc^R, *srl-1(?)*, *dnaA204*	J.Wechsler	

Table 2.1b. Bacterial strains other than *E.coli*

species	strain	source	reference
Alcaligenes faecalis	1053	Joan Fleming	
Azotobacter vinelandii	NCIB 8789	Joan Fleming	Stone & Wilson, 1952
Chromobacterium violaceum	NCIB 9131	Joan Fleming	Sneath, 1956
Citrobacter freundii	NCTC 9750	Joan Fleming	Judicial Commission, 1963
Enterobacter aerogenes	NCTC 10006	Joan Fleming	Hormaeche & Edwards, 1958
Enterobacter cloacae	153	Joan Fleming	Jack & Richmond, 1970
Hafnia alvei	NCTC 6578	Joan Fleming	Gale & Epps, 1943
Klebsiella pneumoniae	NCTC 5055	Joan Fleming	Gastings & Snijders, 1937
Morganella morgani	NCTC 235	Joan Fleming	Morgan, 1906
Proteus vulgaris	NCTC 4175	Joan Fleming	Ekladius *et al*., 1957
Providencia alcalifaciens	ATCC 9886	Joan Fleming	Buchanan, 1962
Providencia stuartii	1190	Joan Fleming	Smith *et al*., 1976
Pseudomonas aeruginosa	PA02	P. Meulien	
Pseudomonas putida	AC34	P. Mieulien	
Rhizobium leguminosarum		J. Lamb	
Serratia marcescens	NCTC 1377		Monsour & Colmer, 1952
Vibrio natriegens	NCMB 857 His-	Frances Pace	Baumann *et al*., 1971

Table 2.2: Phage and Plasmids

Phage

Phage	Genotype	Source/Reference
P1kc		Laboratory stock/Newman & Masters, 1980
λcI-	cI	N.E.Murray
λvir		Laboratory stock
λi21cI-	imm21, cI	N.E.Murray
λcI857	cI857, Sam7	Laboratory stock
λ616	lac5, att+, imm21, cI+ ninR5	Laboratory stock, Wilson & Murray, 1979
λ425	tna+, dnaA+, sr1(1-2) att, int, imm21, ninR5	A. Wright Schaus *et al.*, 1981a
λEH11	insertion of 9.8kb EcoR1 fragment carrying oriC in λ616	Laboratory stock, Hinchliffe *et al.*, 1983

Plasmids

plasmid	genotype	Source/Reference
pBR325	bla, cat, tet	Laboratory stock Bolivar, 1978
pACYC184	cat, tet	N.S.Willetts Chang & Cohen, 1978
pCM959	asnA, oriC	Laboratory stock Meijer *et al.*, 1979
pED935	pBR325, blaΩ(3.608kb::R46tra23kb)	N.S.Willetts Brown & Willetts, 1981
pED936	As pED935	
pHR3	pBR322, tetΩ(0kb::K-12rplL'-rpoC10.5kb)	R.S.Hayward Newman & Hayward, 1980
pSC101	tet	Laboratory stock Cohen & Chang, 1973

Table 2.3 Growth Media

L-broth (LB)

Difco Bacto Tryptone	10g
Difco Bacto Yeast Extract	5g
NaCl	5g

Distilled water to 1l; pH to 7.2 with NaOH

L-broth agar

LB + 15g Difco agar per litre

BBL Agar

Baltimore Biological Laboratories

Trypticase	10g
NaCl	5g
Difco agar	10g

Distilled water to 1l

BBL Top Agar

As for BBL agar, but only 6.5g Difco agar per litre

VB Minimal Agar (Vogel and Bonner, 1956)

1.5% Difco agar in distilled water	400ml
20 x VB salts	25ml
20% carbon source	5ml

Amino acids and vitamins as required

VB minimal medium

20 x VB salts	25ml
20% carbon source	5ml
Sterile distilled water	400ml

Amino acids and vitamins as required

20 x VB salts

$MgSO_4.7H_2O$	4g
Citric acid	40g
KH_2PO_4	200g
$NaNH_4.HPO_4.4H_2O$	70g

Distilled water to 1 litre

Table 2.3 continued

Amino acid and vitamins

Amino acids	20µg/ml
Glutamine, asparagine	100µg/ml
Vitamins	2µg/ml
Uracil	5µg/ml
Thymine	50µg/ml

MacConkey Agar

Difco Bacto MacConkey agar base	40g

Distilled Water to 1 litre
25ml of filter sterilised 20% carbon source added after autoclaving

Burke's Medium (for Azotobacter vinelandii)

K_2HPO_4	0.64g
KH_2PO_4	0.16g
NaCl	0.20g
$CaSO_4.2H_2O$	0.05g
$NaMoO_4$	1mg

Distilled water to 800ml
Autoclave and add:

Ferric Citrate	3mg
$MgSO_4$	0.20g

pH adjusted to 7.2, add glucose or other carbon source to 2%, water to 1L and agar to 1.5% if required.

TY Medium (for Rhizobium leguminosarum)

Difco tryptone	20g
Difco yeast extract	12g
Calcium chloride	5.3g
Distilled water	4 litres

For plates, add 3.5g agar/200ml

BHI Medium (for Vibrio Natriegens)

Difco Brain Heart Infusion	37g
Nacl	25g
Distilled water	1 litre

Table 2.4 Buffers

Phage Buffer

Na_2HPO_4	7g
KH_2PO_4	3g
NaCl	5g
$MgSO_4$, 0.1M	10ml
$CaCl_2$, 0.01M	10ml
Gelatin solution, 1%	1ml
Distilled water to 1 litre	

Phosphate-buffered Saline (PBS)

KH_2PO_4	30g
Na_2HPO_4	70g
NaCl	40g
$MgSO_4.7H_2O$	2g
Distilled water to 10L	

Tris-EDTA (TE) Buffer

0.01M Tris/HCl

1mM EDTA

TEN Buffer

TE buffer + 5mM Nacl

1 x SSC

0.15M NaCl

0.03M trisodium Citrate

2.3 Bacterial techniques.

Growth of bacteria: Bacterial cultures were routinely grown with shaking at 37°C in LB.

Selection of antibiotic resistance: The routine concentrations and storage condition for antibiotics are given in table 2.5. Where low concentrations of antibiotic were used (5mg/ml or less) the antibiotic was made up fresh on the day of use and plates were not stored.

All antibiotics were used in complex or minimal media as required, except trimethoprim which was used only in minimal medium.

Measurement of Optical Density: Optical density of cultures was measured in a Perkin-Elmer Coleman model 55 spectrophotometer at 540 or 650nm.

Measurement of Cell Size and Cell Number: cell size distribution and cell number were measured in a Coulter Counter Model ZB interfaced with a Coulter Channeliser attached to an XY Recorder II chart recorder (Coulter Electronics Ltd., Harpenden, England). 0.5ml samples of cultures were mixed with 0.5ml 20% formaldehyde in bacterial buffer. Samples of 50 - 200µl as appropriate were diluted into 8ml of filtered azide-saline solution (NaCl, 36g;NaN_3, 2g;distilled water to 4l) and counted. Cell number, median cell volume and modal cell volume were recorded.

Test for Ultraviolet sensitivity of polA strains

Liquid cultures were grown to late-exponential or stationary phase, and a loopful was innoculated as single streak across an L-Broth plate. Half of the streak was exposed to 400 $ergs/mm^2$ of UV from a germicidal lamp. Where more accurate determination of sensitivity was

Table 2.5. Antibiotics

Antibiotic	routine concentration in media (μg/ml)	Stock concentration (mg/ml)	solvent for stock solution	storage temperature °C
Tetracycline	10	12.5	50% EtOH/Water	-20
Ampicillin	50	100	Water	-20
Chloramphenicol	20	20	EtOH	4
Streptomycin	100	20	Water	4
Naladixic Acid	20	4	0.2N NaOH	4
Trimethoprim	50	10	Methanol	4
Kanamycin	15	10	Water	4

required, an "exposure gradient was used. This was achieved by placing a piece of card over the plate, and moving it at set intervals (usually twenty seconds) to expose successively larger segments at each interval.

<u>Hfr-mediated plasmid conduction:</u> the method was essentially that of Yamada and Hirota (1982). Hfr donor strain transformed with plasmids were grown up at 37°C o/n in LB+tet, diluted 20 fold in LB and grown to an OD_{540} of 0.5 as a static or very gently shaken culture in a flask of 10x the culture volume to maximise aeration. 10ml of StrS donor culture was mixed with 1ml of StrR recipient culture which had been treated similarly, and after 30 or 60 mins., the mixture was vortexed vigorously for 1min and plated on minimal medium selective for an early transferred marker, and LB+tet, both containing streptomycin.

<u>Filter mating technique for R46 - mediated conjugation:</u> R46 is a stiff pilus plasmid and efficient conjugation via its transfer system requires a solid support, therefore matings using R46 derivatives were done on membrane filters.

Donor and recipient cultures were grown to OD_{540} = 0.5; 0.2ml of donor and 1.8ml of recipient were mixed and the suspension was filtered through a sterile 25mm (0.45µm diameter pore size) millipore nitrocellulose membrane filter in a Swinnex filter assembly using a 2ml syringe.

The filter was incubated on a prewarmed LB plate at 37°C for 30 mins then removed to a 25ml vial and the mating mixture was resuspended in 2ml LB by vortexing and plated on appropriate selective plates.

Curing of Tn10 by selection of TetS strains:(Bochner et al.,1979) Approximately 10^7 cells were plated on the following medium:

Agar 15g

Bacto Tryptone 10g

Yeast Extract 5g

NaCl 10g

$NaH_2PO_4.4H_2O$ 10g

Glucose 2g

Chlortetracycline HCl 50mg

H_2O 1 litre

Autoclaved 20 minutes and quinaldic acid added to 200μg/ml.

Autoclaved chlortetracycline is non-toxic, but induces the tetA gene and thus renders cells hypersensitive to the lipid-soluble chelating agent quinaldic acid. All colonies (5/5) tested which formed on this medium were tetracycline sensitive.

Strain monitoring: the identity of bacterial strains other than E.coli was checked using the API 20E system (API System S.A., La Balme Les Grottes, 38390 Montalieu Vercieu, France) according to the manufacturers' instructions. The results were analysed using the percentage positive reaction for each species for the 20 tests supplied by the manufacturers, and a 'fit probability' for each species was calculated as shown in the example below.

Test	ONPG	ADH	LDC	
Result	+	-	+	
% +ve	71	2	100	(for Hafnia alvei)

A value of 0.71 x 0.98 x 1 would be calculated as the "probability" that this strain is Hafnia alvei. Values of 0.01 to 0.4 were found for the twenty tests in most cases, confirming the identity of the strains. The exceptions were the two Providencia strains for which values of $<10^{-5}$ were obtained. These strains did not however give a better fit

to any of the strains in the list and so are included and listed under their given names.

These tests were not applicable to Rhizobium leguminosarum, which was identified on the basis of its bone-white and shiny colony morphology (J.Lamb, personal communication); Azotobacter vinelandii, for which growth on nitrogen-free medium was considered sufficient proof of identity; or Chromobacterium violaceum, whose intense inky-violet pigmentation was sufficient identification.

2.4 Phage Techniques.

Preparation of P1 plate lysates: 10^7 pfu of P1 and 0.1 ml of an overnight or late log phase culture were mixed and incubated at room temperature for 20'. The mixture was mixed with 2.5ml BBL top agar plus 1mM $CaCl_2$ and poured onto a freshly made LB+1mM $CaCl_2$ plate. After incubation at 37°C for 8hrs, or overnight, the top layer was scraped off and vortexed with 1ml of LB+1mM $CaCl_2$ and a drop of chloroform. The suspension was centrifuged at maximum speed in a bench centrifuge and the supernatant was removed and titrated. P1 was routinely titrated on W3110 which was found to give larger plaques than most other strains.

P1 transduction: recipient cultures were grown up to late log or stationary phase in LB+1mM $CaCl_2$. Unless strains were lysogenic for P1, they were concentrated 10x by pelletting in a bench centrifuge and resuspension in 1/10 volume of LBC. 0.1ml of undiluted lysate or 2-3 x 10^7 pfu (where precise transduction frequencies were to be measured) were added and the mixture was incubated at room temperature for 20 mins. Where antibiotic resistance was to be selected, 1ml of LB was added to the mixture and it was incubated at 37°C for 30 mins before plating. Otherwise, 1ml of phage buffer was added and the mixture was plated on selective media. For the selection of some nutritional markers it was found to be necessary to wash and resuspend the transduction mix in buffer + 200mM trisodium citrate before plating, and this procedure was routinely adopted in later experiments.

Selection and testing of λ lysogens: a late log phase culture was mixed with BBL top agar + 10mM $MgSO_4$ and poured onto an LB plate. 10-50µl of phage suspension was spotted on and the plate was incubated overnight. Bacteria growing within the phage spot were streaked out and incubated overnight and single colonies were tested for lysogeny by cross streaking against λcI- and λvir for imm^{21} lysogens. For imm^{21} lysogens, λimm^{21}cI- was used in place of λcI-. Lysogens were repurified and retested before use.

Growth of Phage λ.

Plate lysates: these were prepared as for P1, except that a single fresh plaque vortexed in 1ml of phage buffer, with a drop of chloroform was used as the source of phage innoculum wherever possible, and 10mM $MgSO_4$ was substituted for $CaCl_2$.

UV induction of λ lysogens: lysogenic bacteria were grown to early log phase (OD_{540}=0.5), harvested by centrifugation and resuspended in 1/2 the original volume of 10mM $MgSO_4$. The cells were exposed to 400ergs/mm^2 of UV light in 10cm glass petri dishes, then diluted 4x into fresh, prewarmed LB supplemented with 10mM $MgSO_4$, and grown in the dark at 37°C for 2 hours or until lysis was observed. To achieve maximum aeration, the culture was incubated in a vessel no less than 10 times the volume of the culture and shaken as vigorously as possible. Chloroform was added and the lysate was clarified and titrated

Preparation of λ phage by induction of thermoinducible lysogens: overnight cultures were grown at 30°C and diluted 50x in LB. When an OD_{540} of 0.5 had been reached, the culture was transferred to a 42°C waterbath and shaken for 20 minutes to induce the prophage. The culture was then transferred to 37°C and shaken until lysis occurred (approximately 2 hours) when chloroform (5ml/250ml) was added. Phage were concentrated as described below.

Amplification of λ phages by liquid infection.

a) Single cycle lysates: 1 litre of *E.coli* C600 was grown to an OD_{540} of 0.5 in 250ml lots in 2 litre flasks with vigorous shaking; λ phage to an m.o.i. of one and glucose to 0.2% were added. The culture was further incubated with vigorous agitation for 2-3 hours, or until lysis was visible. Phage were concentrated as below.

b) Multiple cycle lysates (Maniatis et al 1982): C600, JF50 (which was used for the preparation of λ425, since C600 was found to give very low titres with this phage) or SM32 was grown up overnight and the optical density measured. A volume of culture equal to 12.4ml divided by OD_{600} was harvested by centrifugation and resuspended in 3ml of phage buffer. Phage (5×10^7) were added and the mixture was incubated with shaking at 37°C for 20 minutes. The mixture was added to 500ml prewarmed LB and incubated with vigorous shaking at 37°C for 10-11 hours. 10ml of chloroform was added and the culture was incubated for a further 30 minutes, then DNase and RNase were added to 1μg/ml and the lysate was incubated for a further 30 minutes. Solid NaCl was added to give a final concentration in solution of 1M and the lysate stood on ice for 1 hour. Cell debris was removed by centrifugation at 11,000g for 10 minutes at 4°C. The supernatant was decanted and PEG 6000 was dissolved by gentle stirring with a magnetic stirrer at room temperature and the mixture was left overnight at 4°C. The PEG precipitate was harvested by centrifugation at 11,000g for 10 minutes, the supernatant decanted off and the pellet drained. The pellet was resuspended in 8ml phage buffer with a wide bore pipette fitted with a rubber bulb, then the PEG was removed by vortexing the suspension with an equal volume of chloroform and separation of the phases by centrifugation at 1600g for 15 minutes at 4°C. The aqueous layer containing the phage was loaded onto a CsCl step gradient and purified as described below.

Concentration of phage:

a) By PEG precipitation (Yamamoto et al., 1970): Phage lysates were prepared and clarified as above and DNase (DNase1, Sigma) and RNase (RNaseA, Sigma) were added to a final concentration of 10μg/ml. The lysate was incubated at room temperature for 1 hour and then NaCl (40g/l) was added and dissolved by stirring. PEG 6000 was added to a concentration of 10%, dissolved by stirring and the lysate was left overnight at 4°C. The precipitated complex of PEG and phage was harvested by centrifugation at 10,000 rpm for 15mins in a GSA rotor of a Sorvall RC5B centrifuge, and the pellets were resuspended in about 1/50 of the original volume by rotary shaking at 4°C for a few hours. Debris was sedimented by low speed centrifugation.

b) By pelletting: Phage lysates were clarified and treated with DNase and RNase as above. They were then centrifuged at 20,000 rpm in a type 21 rotor in an MSE Superspeed 65 ultracentrifuge for 3hrs. Pellets were resuspended in 1/50 of the original volume of phage buffer by gentle rotary shaking at 4°C. Debris was removed by centrifugation in a bench centrifuge.

Caesium chloride step gradients: Phage suspensions concentrated by either of the two above methods were loaded onto CsCl step gradients, composed of three 1.5ml steps of density 1.7, 1.5 and 1.3 g/cm^3. The gradients were centrifuged at 22,000 rpm in an AH627 (swing out) rotor of a Sorvall OTD50 ultracentrifuge for 3 hours. The phage bands were removed with a syringe fitted with a 23 gauge needle. Phage for use as size markers were used directly from the step gradient for the preparation of DNA. For cloning and hybridisation purposes, they were further purified by equilibrium centrifugation.

Caesium chloride equilibrium gradients: Phage bands were mixed with caesium chloride and adjusted to 1.5g/cm^3 in phage buffer, and loaded

into 15ml polyallomer heatseal tubes. Gradients were formed by centrifugation in a Ti50 rotor in a Sorvall OTD50 centrifuge for 30 hours at 40,000 rpm. Phage bands were removed as above. Phage were stored at 4°C in CsCl until required for use.

2.5 Measurement of DNA synthesis in Thy+ strains

Overnight cultures growing in LB were subcultured into LB+200μg/ml uridine, 6.5μg/ml thymidine, and 25μCi/ml ^3H thymidine (49Ci/mMol, obtained from Amersham International) and grown for at least 3 generations at 30°C. The culture was then diluted into the same medium at 30°C and 40°C. 50μl samples were removed at intervals onto 1cm squares of Whatmann 3MM filter paper and labelling was stopped and the nucleic acids precipitated by immersing the filters in 7.5% trichloroacetic acid. Filters were washed 3 times in 7.5% trichloracetic acid and once in 95% ethanol and dried at 42°C. Radioactivity was estimated by liquid scintillation counting using a scintillation fluid containing 0.5% butyl BPD in toluene in a Packard Tricarb scintillation counter.

The growth of cultures during labelling was followed by measuring absorbance in a Klett colorimeter.

2.6 DNA Techniques

Extraction of phage DNA: Phage concentrated in CsCl was dialysed against three changes of TE buffer over 12hrs, transferred to a polypropylene snapcap tube and extracted three times with redistilled phenol equilibrated against TE buffer. The aqueous layer was removed and dialysed against 3 changes of TE buffer over 24 hours. DNA was stored in TE buffer at 4°C.

Small scale preparation of λ DNA from lysogens: 25ml cultures of λ lysogens were grown up and UV-induced as above (2.4). The lysate was clarified and the phage were pelleted in 30 ml polycarbonate tubes at

19,000 rpm in a SS34 rotor of a Sorvall RC5B centrifuge. The pellets were resuspended in 1ml of phage buffer and the suspension was transferred to a 1.5ml microfuge tube. The suspension was centrifuged for 2 minutes to remove debris and the supernatant was extracted 3x with an equal volume of phenol and once with 24:1 chloroform/isoamyl alcohol and the nucleic acids in the aqueous layer were precipitated with 2 volumes of ethanol, after the addition of 1/10 vol of 3M sodium acetate - 0.1M magnesium acetate, at -20°C for 30 minutes. The nucleic acid was harvested by centrifugation and the pellet was dried and resuspended in 50µl of TE buffer. 25µl of this was found to be sufficient for one gel.

This method has advantages over other methods for rapid small scale preparation of λ DNA which mostly rely on plate lysates. It is quicker than the plate lysate method of Cameron _et al_. (1977), does not involve the use of agarose (which sometimes contains sulphated sugars which inhibit restriction enzymes), and it is applicable to phage prepared by heat or UV induction, as well as by infection. Pelleting of phage in a high speed centrifuge obviates the need to use an ultracentrifuge. It is quicker than PEG precipitation and the pellet is easier to process.

Preparation of Bacterial DNA: 250ml bacterial cultures for DNA extraction were grown up overnight, or in the case of slow growing strains (_Rhizobium leguminosarum_ and _Azotobacter vinelandii_) until the culture had reached an OD_{540} = 1.5 approximately. Cells were harvested by centrifugation and the pellet was resuspended in 50ml 25% sucrose in 50mM Tris buffer pH 8.0 with 1.7ml of lysozyme solution (10mg/ml;Sigma grade 1 from egg white), and placed on ice for 5 minutes. 6.5ml EDTA was added and the mixture was incubated for a further five minutes before addition of Triton lysis mix (2% Triton X100, 0.0625M EDTA, 50mM Tris HCl pH 8.0). After 20 minutes 2.8mg of preincubated protease (Sigma type IV) was added and the mixture was incubated at 37°C for 1 hour. All steps up to this stage were carried out on ice. Where lysis was not complete, longer incubation periods were employed (up to 12hours) after the addition of protease.

The lysate was extracted 3x with an equal volume of phenol in a

30ml acid washed and siliconised Corex tube. The layers were mixed by gentle shaking and the resulting emulsion was separated by centrifugation in an SS34 rotor of a Sorvall RC5B centrifuge at 10,000 rpm at $4^{\circ}C$. The phenolic layer was removed with a pasteur pipette, until the final extraction when the aqueous layer was removed.

DNA was recovered from this solution by layering on two volumes of ice cold ethanol and spooling the DNA that precipitated at the interface onto an acid washed glass rod. The DNA was redissolved in 10mM Tris-HCl pH 8.0, 1mM EDTA, 270mM NaCl and treated with RNase (20μg/ml boiled for 20' to inactivate any DNase activity), then phenol extracted and spooled as above. The DNA was redissolved in TE buffer and dialysed against 3 changes of TE over 24 hours.

Small scale plasmid preparation (Birnboim and Doly, 1979): The solutions used for this preparation are listed in table 2.6.

1.5ml of an overnight culture was pelleted in a 1.5ml microfuge tube at maximum speed in a microfuge. The supernatant was discarded and the pellet was resuspended in 0.1ml of lysis solution by vortexing, and left on ice for 30 minutes. 0.2 ml of alkaline SDS was added and after 5 minutes, 0.15 ml of high salt. The suspension was mixed by inversion, then left on ice for 60 minutes with occasional mixing. The precipitate was removed by centrifugation for 5 mins at maximum speed in a microfuge and the supernatant transferred to a fresh microfuge tube and 1ml of ice cold ethanol added. The mixture was left at $-20^{\circ}C$ for 30 minutes and the precipitated nucleic acids were pelleted by centrifugation in a microcentrifuge for 2 minutes. The supernatant was discarded and the pellet dissolved in 0.1ml of 0.1M sodium acetate, pH6. 200ml of ethanol was added and the nucleic acids precipitated at $-20^{\circ}C$ for 10 minutes and pelleted by centrifugation for 5 minutes and, after removal of the supernatant, dried under vacuum. The dried pellet was dissolved in 50μl of TE buffer.

Table 2.6 Solutions for Birnboim Preparation.

Lysis solution	
Lysozyme	2mg/ml*
Tris/Hcl pH 8.0	25mM
EDTA pH 8.0	10mM
Glucose	50mM
Alkaline SDS solution	
NaOH	0.2M
SDS	1%
High Salt Solution	
Sodium acetate	3M
to pH5 with acetic acid	
Low Salt solution	
High salt solution diluted 1:30	

* It was found during the course of experimentation that lysozyme could be omitted from this solution without significantly reducing the yield of DNA

Large scale preparation of plasmid DNA.

Method 1: For ColE1 replicons. A 500ml overnight culture in LB + appropriate antibiotics was harvested by centrifugation and the pellet was resuspended in 6ml 25% sucrose, 50mM Tris-HCl pH 8.1, 40mM EDTA. 1ml of 10mg/ml lysozyme in 50mM Tris/HCl pH 8.1, 40mM EDTA and 1ml of 0.5M EDTA pH 8.1 were added and the mixture was swirled gently and placed on ice for 5 minutes. 13ml of Triton lysis mix (2ml 10% Triton X100 25ml 0.5M EDTA, 10ml 1M Tris/HCl pH 8.1, H_2O to 200ml) was added and the mixture was swirled on ice for 10 minutes during which period lysis occurred. The lysate was clarified by centrifugation at 15,000 rpm for 30 mins at 4°C in an SS34 rotor of a Sorvall RC5B centrifuge. The non-viscous supernatant was decanted and 0.95g CsCl and 0.1ml 5mg/ml ethidium bromide were added. The solution was loaded into 13ml polypropylene heatseal tubes and subjected to equilibrium density gradient centrifugation at 38,000 rpm in a Ti50 rotor of a Sorvall OTD50 centrifuge for 2-3 days at 15°C. The plasmid-ethidium bromide band was visualised by illumination under long wave UV light and collected by side puncture with a 2ml syringe fitted with a 23 gauge needle. Ethidium bromide was removed by extracting 3x with an equal volume of isoamyl alcohol (equilibrated with saturated CsCl solution) and the solution was dialysed against 3 changes of 500ml TE buffer over a period of 24 hours to remove CsCl.

Method 2: for low copy number plasmids. (After Humphreys et al, 1975) 2l of culture were harvested and a cleared lysate was prepared as in method 1 except that the quantities of reagents were increased x4. 3% NaCl and 10% PEG 6000 were added to the cleared lysate and dissolved by inversion. The solution was transferred to a 50ml centrifuge tube and left at 4°C overnight for the PEG-plasmid complex to form. The PEG precipitate was pelleted at maximum speed in a bench centrifuge for 2 minutes, drained, dissolved in 7ml of TES buffer (50mM Tris/HCl, 5mM

Na_2EDTA, 50mM NaCl pH8.0) and transferred to an acid washed and siliconised Corex tube. 11.0g CsCl and 0.5ml of 10mg/ml ethidium bromide were added. The mixture was stood on ice for 30 minutes and the flocculant precipitate of PEG was removed by centrifugation at 10,000 rpm for 30 minutes at 4°C. The plasmid solution was removed from below the pellicle of precipitated PEG with a pasteur pipette and a further 0.2ml of ethidium bromide was added. The density of the solution was adjusted to 1.5g/cm^3 and the solution was subjected to equilibrium centrifugation as above in 13ml Beckman Ultraclear centrifuge tubes fitted with metal caps. Where no plasmid band was visible under long wave UV illumination, the gradient was fractionated using an MSE fractionator. Visible plasmid bands were removed as in method 1. Subsequently, fractions were treated as in method 1.

Method 3: For low copy number plasmids. (After Hansen and Olsen, 1978): a 2l culture was grown up overnight under selection and harvested by centrifugation for 10 minutes at 9,000 rpm in a GS3 rotor of a Sorvall RC5B centrifuge. The pellets were resuspended in 60ml of 20% sucrose in TE buffer and 5 mls of lysozyme (10mg/ml) and 20 ml of 0.25M EDTA pH 8.0 were added. After 5 minutes, 25ml of 20% SDS in TE buffer was added and the lysate was subjected to 8 heat pulses of 15 seconds at 55°C alternated with 15 seconds of mixing by inversion. DNA was denatured by the addition of 24mls freshly prepared 3M NaOH to bring the pH to 12-12.5. Closed-circular DNA was renatured by the addition of 40ml saturated Tris-HCl pH 7.0 to obtain a pH of 8.5-9.0, and single stranded DNA was precipitated by the addition of 32ml 20% SDS in TE and 64 ml 5M NaCl. The solutions were mixed by inversion and placed at 0°C for 12 hours.

The salt precipitate was removed by centrifugation for one hour at 11,000 rpm in a GSA rotor of a Sorvall RC5B centrifuge and the plasmid DNA was precipitated by the addition of 1/4 volume of 25% PEG 6000 solution. The suspension was left at 4°C for 6 hours and the PEG precipitate was harvested by centrifugation at 5,000rpm for 5 minutes. The precipitate was

processed and plasmid DNA was prepared as described in method 2.

Determination of Densities of gradient fractions: 200 or 400µl samples were collected with a Gilson Pipetman P200 or p1000 automatic pipette, transferred to a tared microfuge tube, and weighed on a Mettler H10T microbalance. Fractions were allowed to equilibrate to room temperature (21 +/- $3^{\circ}C$) before sampling.

Ethanol Precipitation of Small Quantities of DNA: 1/10 volume of 3.0M sodium acetate, 0.1M magnesium acetate was added to DNA in TE and 2 volumes of ethanol were then added. The nucleic acid was allowed to precipitate for 1 hour or more. DNA was pelleted in a microcentrifuge, the supernatant decanted and the pellet redissolved in TE buffer.

Restriction of DNA: DNA was digested with restriction enzymes (2-10u/µg DNA) for 1 hour at $37^{\circ}C$ (the exception was SmaI, which was found to be more active at $30^{\circ}C$. Except where otherwise stated, all digestions were performed in universal buffer (see table 2.7) . Reactions were stopped by incubating at 70° to inactivate the enzymes and melt the cohesive ends of λ, which was routinely used (digested with HindIII) as a size marker. For digestion of genomic DNA, the reaction times were often extended to 12 hours to obtain complete digestion.

Ligation of DNA fragments: Ligation reactions were performed at a DNA concentration of 10-50 ng/µl of vector DNA and DNA to be cloned. 1/10 volume of ligase cocktail were added to the reaction mix after heat inactivation of restriction enzymes, and 15 units of T4 DNA ligase was added. The ligation mixture was incubated at $10^{\circ}C$ for 4-10 hours before transformation.

Transformation of E.coli cells. (After Lederberg and Cohen, 1974): Cells competent for transformation were obtained by growing the host

Table 2.7. Buffers for restriction and ligation of DNA

10x EcoRI Restriction Buffer

Component	Concentration
Tris/HCl pH 7.5	1.0M
$MgCl_2$	50mM
NaCl	500mM
2-mercaptoethanol	60mM

10x Universal Restriction Buffer (O'Farrell et al.,1980)

Component	Concentration
Tris/acetate pH 7.9	33mM
Magnesium acetate	10mM
Potassium acetate	66mM
Dithiothreitol	5mM
Bovine serum albumen	1mg/ml

10 x ligase cocktail

Component	Volume
1M Tris/HCl pH 7.2	660ul
0.1M EDTA	100ul
1M $MgCl_2$	100ul
1M dithiothreitol	100ul
0.1M ATP	10ul

distilled water to 1ml

strain to an OD_{540} of 0.5-0.65 in 50ml of LB at 37°C. Cells were chilled on ice for 15 minutes then pelleted and resuspended in 25ml of ice cold 0.1M $MgCl_2$ then immediately repelleted in 2.5ml of ice cold 0.1M $CaCl_2$ and kept on ice for at least 30 minutes. For some strains, the $MgCl_2$ wash was replaced with $CaCl_2$ but this was found to be unsatisfactory for strains carrying polA mutations. Where appropriate, competent cells were stored at -70°C in 20% glycerol, 50mM $CaCl_2$

Transforming DNA was added to 200µl of competent cells and left on ice for 30 minutes, then the mixture was heat shocked for 2 minutes at 42°C and diluted with 1ml of L-broth and incubated at 37°C for 30 minutes before plating to allow expression of antibiotic resistance, then spread on selective plates.

In vitro packaging of λ DNA: EcoR1 libraries in λ vectors, and all other cloning reactions involving λ vectors were introduced into E.coli by in vitro packaging. Buffers used for this reaction are shown in table 2.8.

Preparation of freeze-thaw lysate (FTL):

3 x 500ml cultures of BHB2688 met-(λimm^{434},cIts62,red3,Eam4,Sam7)/λ+ were grown up to OD_{650} of 0.3 at 30°C in LB and induced by placing the flasks in a 45°C waterbath for 15 minutes. The cultures were incubated with vigorous shaking at 37°C for 1 hour and pelleted by centrifugation in a 6 x 250 ml rotor at 9,000 rpm for 10 minutes. The pellets were thoroughly drained and resuspended in a total of 6ml of cold 10% sucrose in 50mM Tris-HCl pH 7.5 with a pipette. The suspension was dispensed into 2 10ml polycarbonate ultracentrifuge tubes. 75µl of fresh lysozyme solution (2mg/ml in 0.25M Tris-HCl pH 7.5) was added to each tube and the mixture was frozen quickly in liquid nitrogen and stored at -70°C. The suspension was thawed on ice then 75µl of buffer M1 was added to each tube. After thorough gentle mixing the lysate was centrifuged at 35,000 rpm for 35 minutes. The supernatant was dispensed in 100µl aliquots, frozen in liquid nitrogen and stored at -70°C.

Table 2.8. Buffers for in vitro Packaging

Buffer A

20mM Tris/HCl pH8

3mM MgCl2

0.05% v/v 2-mercaptoethanol

1mM EDTA

Buffer B

110µl H_2O

6µl 0.5M Tris/HCl pH7.5

300µl 50mM spermidine,100mM putrescine neutralised with Tris base

9µl 1M $MgCl_2$

75µl 0.1M ATP neutralised with NH_4OH

1µl 2-mercaptoethanol

Preparation of sonicated extract (SE): 500ml of BHB2690 met-,(λ imm^{434},cIts62,red3,Dam15,Sam7)/λ was grown up to OD650 of 0.3 and induced and harvested as described above for the freeze-thaw lysate. The pellet was resuspended in 4.6ml of buffer A and transferred to a plastic tube. The suspension was sonicated in 3 second bursts at maximum setting in an MSE 100W ultrasonic disintegrator until no longer viscous. Debris was pelleted at 6,000 rpm for 6 minutes and the supernatant was quick frozen in 50μl aliquots in liquid nitrogen and stored at -40°C

For the packaging reaction, FTL and SE were thawed and a packaging mixture prepared by mixing the following materials in the order given: 7μl buffer A, 2μl DNA, 1μl buffer M1, 4-8μl sonicated extract, 10μl freeze-thaw lysate. The reaction was incubated at 25°C for 60 minutes and diluted into 0.5ml of phage buffer. The titre of the packaged phage mixture was determined and a plate lysate prepared as described above under phage methods.

DNA electrophoresis on agarose gels (McDonnell et al., 1977): for a horizontal, 0.7%, 25 x 15cm gel, 1.4g of agarose (Sigma type1, low EEO) was dissolved in 200ml Tris-acetate buffer (40mM Tris, 20mM sodium acetate, 1mM EDTA, pH 8.2 with acetic acid) by boiling for 2 minutes. After cooling to 50-60°C, Ethidium bromide (0.5 μg/ml) was added and the gel was poured in a 15 x 25cm Perspex gel former with a slot former giving 1cm wide 30μl capacity wells placed 5cm from one end. The gel was allowed to set for 1 hour then the slot former was removed and the gel was either immersed in an electrophoresis tank containing Tris-acetate buffer or flooded with the same buffer before loading.

20-25μl of DNA was mixed with 5μl of loading buffer (50% glycerol, 1x Tris-acetate buffer, 0.04% bromophenol blue) and loaded into the wells. Electrophoresis was at 100V overnight, either in the electrophoresis tank or with blotting paper wicks dipping into electrode

tanks containing Tris Acetate buffer. Bands were illuminated with UV light and photographed using Ilford FP4 film with a 15 second exposure and a red filter.

Extraction of DNA from agarose gels. DNA fragments were separated by electrophoresis as described above except that wider wells (typically 2cm) were employed to allow the loading of more sample. Enough DNA to give approximately 1ug of fragment was loaded onto the gel. Bands were visualised by ethidium bromide staining and ultraviolet illumination and the relevant portions of the gel were cut out with a clean razorblade.

The pieces of DNA-containing agarose were placed in a 1.5ml microfuge tube and 3 volumes of saturated KI in 10mM pottassium phosphate pH 6.8 were added. The tube was vigorously vortexed and placed at 37°C for 1hr or until the agarose had dissolved. DNA was isolated from this solution by hydroxyapatite chromatography as below.

Isolation of DNA by hydroxyapatite chromatography: Hydroxyapatite (HAP, Bio-Rad DNA grade, Bio-gel HTP) was shaken with distilled water and allowed to settle out, then the supernatant was decanted off to remove fines. This was repeated 3 times. The aqueous slurry of HAP (about 200µl was loaded into a 1ml pipette tip plugged with sterile siliconised glass wool.

The column was then washed with 3ml of distilled water then 3ml of saturated KI in 10mM potassium acetate pH 6.8 and the dissolved gel slice in saturated KI was loaded onto the column, which was washed with a further 3ml of saturated KI. The KI was washed from the column with 3ml of 50mM Na_2PO_4 pH 6.8.

DNA was eluted from the column by passing 400µl of 400mM Na_2PO_4 pH 6.8 through and collecting 3 drop fractions. The DNA usually eluted in the second, third and fourth drops.

The washing and elution of the column was carried out under gentle positive pressure applied with a Gilson Pipetman P1000 automatic pipette.

Phosphate salts were removed from the DNA solution by passing it down a 7ml column of Sephadex G50 in distilled water and the DNA was concentrated by ethanol precipitation as described above.

DNA-containing fractions were identified by spotting 1µl or 5µl onto an agarose-ethidium plate. This was prepared by dissolving 0.25g agarose in 25ml distilled water and adding 12.5µl of 10mg/ml aqueous ethidium bromide solution and allowing this to set in an 8cm polystyrene petri dish. DNA-containing spots could be identified by their orange fluorescence under ultraviolet illumination.

Labelling of DNA by nick translation: 10-20µCi of ^{32}P-dCTP (The Radiochemical Centre, Amersham International, Bucks.) was dried down under vacuum and resuspended in 10µl of 10x reaction buffer (50mM Tris-HCL pH7.8; 5mM $MgCl_2$; 10mM 2-mercaptoethanol) with 1µl each of 10mM dGTP, dATP, and dTTP, and 1ug of DNA in TE buffer was added. 1µl of DNase 1 (1µg/ml stock solution of Sigma type 1 pancreatic DNase) was added and nicking of the DNA was allowed to proceed at room temperature for 2 minutes. DNA polymerase 1 (1µl of 1u/µl stock solution was added and the reaction was incubated at 15°C for 3-4 hours. At the end of this period, 2µl of orange G dye was added and the DNA separated from the unincorporated nucleotides (which elute with the marker dye) on a 10ml Sephadex G50 column in TEN buffer. 10 drop fractions were collected and counted by Cherenkov counting in a Packard Tri-Carb liquid scintillation counter. Fractions containing labelled DNA were pooled and stored at -20°C unless used immediately.

Where indicated, the probe was further purified on an Elutip column (Schleicher and Schuell), according to the manufacturers' recommended method.

The buffers used were:

Low Salt	High Salt
0.2M NaCl	1.0M NaCl
20mM Tris-HCl	20mM Tris-HCl
1.0mM EDTA	1.0mM EDTA
pH 7.3-7.5	pH 7.3-8.5

The column was washed with 2 ml of high salt, then 5ml of low salt. The DNA solution, in low salt was slowly pumped through the column. Binding was essentially quantitative. The column was then washed with 3ml of low salt and the DNA was eluted from the column with 0.4ml high salt. About 50% of the labelled DNA was found to bind irreversibly to the column.

In spite of the high specific activity of the probe, it was found that this purification seemed to reduce the strength of hybridisation signals. This may be on account of the use of smaller ammounts of DNA (0.25µg rather than 1-2µg) used for the preparation of these probes (see discussion) or the selective removal of that fraction of the probe which most readily reanneals, possibly the larger labelled fragments.

Colony hybridisation: Colonies were screened for the presence of homologous DNA sequences by a modification of the procedure of Grunstein and Hogness (1975). Colonies to be screened were stabbed into an LB agar plate and incubated overnight. The plate was replica plated onto a nitrocellulose filter (Schleicher and Schuell, 0.45µm pore diameter) placed on an LB agar plate and incubated overnight. The filter was then transferred to filter paper pads saturated with the following solutions for the indicated times, with the colony side uppermost.

0.5N NaOH	7min.
1.0M Tris-HCl, pH 7.4	2min
1.0M Tris-HCl, pH7.4	2min

1.5M NaCl, 0.5M Tris-HCl pH 7.4 4min

The filter was then blotted dry and baked under vacuum at 80°C for 2 hours. Conditions for hybridisation were as described for Southern blots.

Southern hybridisations (Southern, 1975)

Transfer of DNA to nitrocellulose Filters DNA was digested and separated in 0.7% agarose slab gels and the gel was photographed. The relevant portions of the gel were cut out, soaked in 0.25M HCl for 2 x 15 minutes to depurinate the DNA and thus improve transfer of large fragments (Wahl *et al.*, 1979), and then soaked in 0.5M NaOH, 1.5M NaCl with gentle shaking for 2 x 20 minutes at 37°C to denature the DNA. The gel was neutralised by gentle shaking in 0.5M Tris/HCl pH 7.4, 3M NaCl for 2 x 20 minutes. The gel was placed on a sheet of blotting paper dipping into a reservoir of 20 x SSC (3M NaCl, 0.3M sodium citrate). A piece of nitrocellulose (Schleicher and Schuell, 0.45µm pore size) cut to the same size and soaked in 2xSSC was placed over the gel, a 2" stack of blotting paper was placed on top of this and the whole stack was evenly weighted. Transfer was allowed to proceed for 12 - 24 hours. The nitrocellulose filter was rinsed in 2 x SSC, blotted dry and baked for 90 minutes in a vacuum oven at 80°C. The gel was restained and examined under UV light to check for complete transfer.

Alternatively, the "dry blot" method of Smith and Summers (1980) was used. The gel was processed as above up to and including the denaturation step, then neutralised in 1M ammonium acetate, 0.02M NaOH for 2 x 30 minutes. A sheet of nitrocellulose cut to size and soaked on 1M ammonium acetate, 0.02M NaOH was placed on top of the gel and 3 sheets of Whatman 3MM filter paper soaked in the same solution were placed on top of this. A 5cm stack of paper towels was placed over this , the stack weighted evenly, and the transfer allowed to proceed for 4 hours at room temperature. The filter was rinsed in 2xSSC and baked as above.

Hybridisations: 0.25 - 1μg of nick translated probe DNA was denatured by incubation at 100°C for 5 minutes, diluted in 10ml of hybridisation solution (1xSSC, 50% formamide). The filter was soaked in hybridisation solution and placed in a sealed plastic bag with the probe solution. The bag was shaken for 12-24 hours at 37°C. The filter was washed in 2xSSC, 0.1% SDS (4 x 1hour changes), then in 2xSSC (2 x 1 hour changes), blotted dry, sealed in a polythene bag, and exposed to X-Ray film (Fuji RX) at -70°C in a Cronex autoradiography cassette fitted with a phosphotungstate intensifier screen.

2.7 Densitometric analysis of hybridisation data.

Autoradiograms and photographs of gels were scanned using a Vitatron TLD100 densitometer attached to a chart recorder. A 100μm slitpiece and a grey (U60) filter were used. The machine was adjusted to give a baseline of 10% and the sensitivity set so that a maximum deflection of 75% was obtained. However, where both very faint and very dark bands were present on an autoradiogram, the sensitivity was increased so that a band which gave measurable deflection on the first scan, gave 75% deflection on the "high sensitivity" scan. Intensities of bands were calculated by integrating the bands by tracing round them on a Summagraphics magnetic data tablet attached to a Ferranti Cetec digitiser and an Olivetti P6040 minicomputer with an area calculation program written by Dr A.Coulsen. Chromosomal DNA, which was not resolved into discrete bands, was estimated in the same way by tracing the area of the "laddered smear". For all values, the average of 3 passes was used as the correct area.

DNA concentration was estimated by dividing the integrated area of the trace of the chromosomal smear by the summed trace areas of the λ standard bands which was taken to be equivalent to 1ug.

In some cases, some bands were undetectable on short exposures, while others were overexposed on long exposures (the latter was usually the case for the E.coli track). In this case, a band whose intensity was

intermediate and measurable on both exposures was integrated and used as an internal standard. All results were standardised to the values for the longest exposure. Thus if the trace area for an internal standard band, $A_{(i)}$ is $A_{(i)l}$ for the long exposure and $A_{(i)s}$ for the short exposure. Intensities calculated from the short exposure are taken as:

$$I = A_{(i)l}/A_{(i)s} \times A_{(m)s}$$

where $A_{(m)s}$ is the the measured intensity of a band on the short exposure.

Intensities are corrected for estimated DNA concentration. Thus the value for band intensity given in the tables in chapter 4 is I(m)/c, where I(m) is the measured intensity of the band (the area of the trace), corrected where necessary for different exposure times; and c is the estimated concentration of chromosomal DNA calculated from the area of the chromosomal trace divided by the area of the λ trace. Thus the units of intensity are in $cm^2/\mu g$. To convert this to % hybridisation, the value of the intensity of all bands in a track has been summed and divided by the value for the fully homologous hybridisation (the <u>E.coli</u> track).

Chapter 3: Characterisation of a suppressor of dnaA

3.1 Introduction

As has been discussed in Chapter 1, the study of suppressors offers a means of elucidating the interaction of genes and gene products in complex processes such as the initiation of DNA replication. For any new suppressor, two pieces of information are of primary interest: the genetic location, which indicates which gene or gene product is causing the suppression; and the allele specificity, which gives basic information on the mode of suppression. If a suppressor acts interactively, it is expected to show a measure of allele specificity.

Analysis of this sort has allowed the classification of certain rpoB mutations as interactive suppressors of dnaA (Schauss et al.,1981b), and of rnh mutations as bypass suppressors which suppress even null alleles of dnaA (Kogoma & von Meyenburg,1983)

In this chapter, the genetic location of a cloned suppressor of dnaA apparently identical to the sdaA suppressor isolated by Takeda & Hirota (1982), together with experiments on the allele specificity aimed at determining the mode of suppression are reported. Other aspects of the effect of the suppressor on cell physiology are also discussed.

3.2 Isolation of λsidA

As a comparison to the hybridisation data obtained with oriC described in chapter 4, it was considered of interest to investigate the conservation of the dnaA gene. Attempts were therefore made to clone this gene into a λ vector. The phage obtained did not however contain dnaA, but an extragenic suppressor of dnaA.

λsidA is recombinant phage apparently complementing the dnaA46(Ts) mutation in E.coli K12. It was isolated from a library of EcoR1 fragments from C600 cloned into the lac+,cI+ replacement vector λNM616. The library was prepared by ligating 300ng of EcoR1-digested C600 genomic DNA and 600ng of λNM616 in a 10μl volume and introduced into the Lac- indicator strain JM101 after in vitro packaging. Titration of the in vitro packaged phage indicated that a total of 9000 pfu were present of which 30% gave white plaques on Xgal (the phenotype of recombinant phage, whose lac gene would be replaced by insert DNA) and thus that the library contained a total of approximately 2600 primary recombinants. The library was amplified by a plate lysate on JM101.

Phage capable of transducing the dnaA46(Ts) strain ED419 to temperature resistance were isolated by two methods:

1) Exponentially growing ED419 cells were mixed with the phage library and plated on LB at 40°C. This approach is limited by the high frequency with which revertants and suppressor mutations of dnaA46 arise, and thus, as table 3.1 shows, the proportion of temperature-resistant clones yielding "complementing" phage is rather small.

2) The library was spotted onto a lawn of exponentially growing ED419 and the plate was incubated at 40°C. In this case, transduction to temperature resistance was clearly observable as a tight cluster of temperature-resistant colonies in the phage spot.

Temperature-resistant colonies were screened for production of complementing phage by treating exponentially growing cultures with chloroform, spotting the killed cultures onto a lawn of ED419, and incubating at 40°C. Enough phage were produced by spontaneous induction to give a positive spot test.

Table 3.1 shows that spot complementation is a more efficient approach to the isolation of complementing phage.

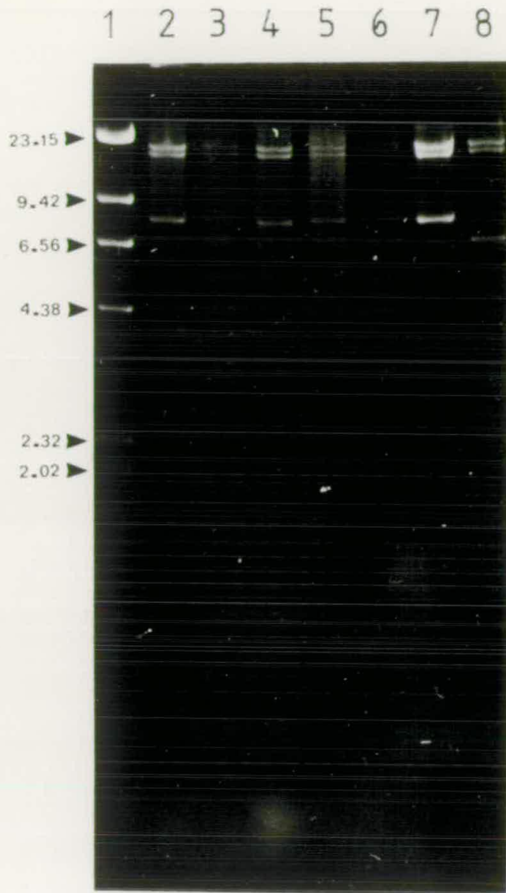
Table 3.1 : Methods of selecting complementing phage

source of temperature-resistant colonies	number of temperature-resistant colonies tested for production of complementing phage	number of initial colonies yielding "complementing" phage
liquid infection (method 1)	12	4
spot complementation (method 2)	10	7

3.3 Screening of Complementing Phage

A new method of DNA isolation was developed for preliminary molecular characterisation of the phage (see Materials & Methods for details). This method, which involves UV induction and pelletting of phage in a high speed centrifuge, yields approximately 1µg of restriction quality DNA from a 25ml culture of a lysogen in only 24hrs. RNA and chromosomal DNA are significant contaminants in this preparation (see Fig. 3.1) but are not present in sufficient quantity to interfere with restriction analysis. Approximately 70% of the phage was recovered in the pelleting step. The method is straightforward, and has the advantage over other rapid methods for phage isolation that it is suitable for lysogens (from which phage cannot be prepared by plate lysate methods) and does not involve the addition and subsequent

Fig. 3.1



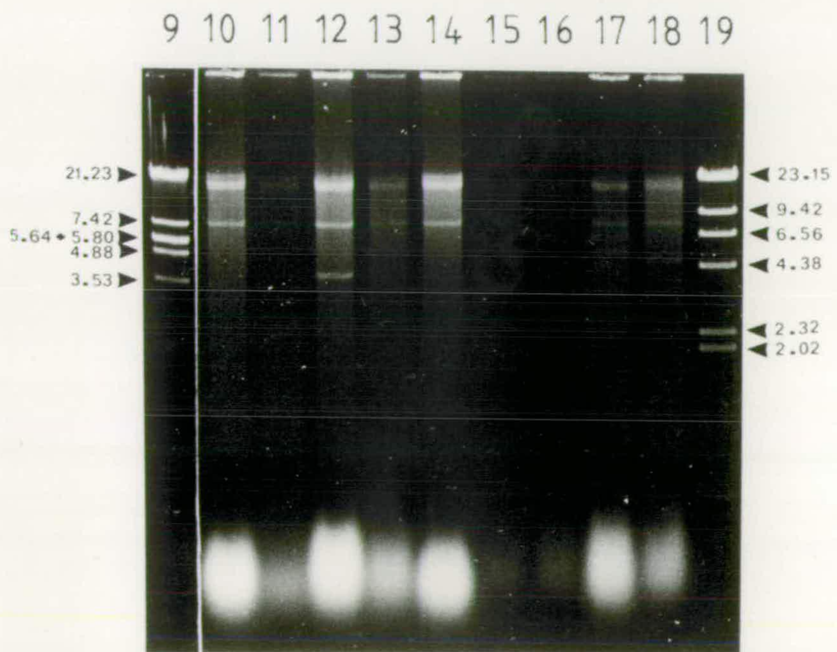


Figure 3.1 : Screening of phage suppressing dnaA

Track	DNA	Enzyme
1	λCI857	HindIII
2	phage no.3	EcoR1
3	phage no.4	EcoR1
4	phage no.5	EcoR1
5	phage no.9	EcoR1
6	phage no.10	EcoR1
7	phage no.2	EcoR1
8	λ 616	EcoR1
9	λcI857	EcoR1
10	phage no.1	EcoR1
11	phage no.2	EcoR1
12	phage no.3	EcoR1
13	phage no.4	EcoR1
14	phage no.5	EcoR1
15	phage no.6	EcoR1
16	phage no.7	EcoR1
17	phage no.8	EcoR1
18	phage no.9	EcoR1
19	λCI857	HindIII

The DNA in tracks 2 - 6 and 10 - 18 was prepared by the rapid method. In addition to the common 8.2kb fragment, additional inserts of 4kb and 4.4kb can be discerned in tracks 2 & 12 and 5 & 18 respectively.

removal of agarose or polyethylene glycol.

All temperature-resistant lysogens screened were found to yield phage with an 8.2 kb insert (see Fig. 3.1). As expected from the fact that the ligation was conducted at a rather high DNA concentration, 2 of the phage (nos. 3 & 9) also contained a second, smaller, insert.

One of the phage (no. 2, Fig. 3.1, track 7) which contained only a single insert was chosen for further study. It was called λ*sidA1* for reasons that will soon become apparent.

A HindIII and EcoR1 restriction map of λ*sidA1* was constructed. Comparison of this with the map of the *dnaA*-containing phage λ425 indicates that the fragment carried by λ*sidA1* does not carry *dnaA* itself (see Fig 3.3).

Furthermore, the restriction map for the *dnaA* region is known (von Meyenburg & Hansen,1980) and the sizes of the EcoR1 fragments surrounding the *dnaA* gene (6.8, 3.6, 6.0, 9.1 kb) preclude the 8.2kb insert in λ*sidA* being from this region. In addition to this, sequence information (Hansen *et al*.,1982) shows that the *dnaA* gene contains an EcoR1 site within its coding sequence which separates the greater part of the structural gene from its promoters. This explains the failure to isolate true *dnaA* containing-phage from the library.

To further confirm that the insert in λ*sidA* was not the *dnaA* gene, Southern hybridisation was carried out between this phage and the *dnaA*-containing phage λ425. No hybridisation was detected between λ*sidA* and the 2.4kb *dnaA*-containing HindIII/XhoI fragment of λ425 (see Fig 3.2)

Since the ability of λ*sidA* to transduce a *dnaA*(Ts) strain to temperature resistance is not due to the presence of the *dnaA* gene itself, the designation SidA (Suppression of Initiation Defect of dnaA) was adopted for the temperature resistance phenotype it confers and the genetic locus responsible for this.

3.4 Is sidA a suppressor indigenous to C600?

Several E.coli strains have been found to carry indigenous suppressors of certain dna(Ts) alleles (Atlung,1981), so to check whether such a suppressor mutation existed in C600, the strain from which the sidA DNA had been isolated, transduction of C600 to dnaA(Ts) was attempted.

The trimethoprim resistance (Tmp^R) transposon Tn7, which has a single chromosomal insertion site in E.coli close to the dnaA gene (Barth et al.,1976), was introduced into ED419 dnaA46 by P1 transduction from J62Tn7 and the resulting dnaA46,Tmp^R strain was used as a donor for P1 transduction of Tmp^R into C600 and R2D2 (a strain which had previously been shown to be capable of transduction to dnaA46(Ts)) to Tmp^R. In both strains, approximately 50% of Tmp^R transductants were temperature-sensitive (see Table 3.2).

Thus C600 does not express the SidA phenotype and so sidA is not an indigenous dnaA suppressor mutation. Instead, it is apparently a piece of wild-type DNA activated to dnaA suppression by its new molecular environment.

3.5 The sidA fragment has the wild-type restriction pattern.

Although sidA is clearly not a chromosomal mutation in the source strain the possibility exists that it is a mutation that has occurred in the sidA phages during propagation. For such a mutation to occur in so many different phages (the presence of different second inserts in 2 phages shows that at least 3 independent recombinants were isolated) would require a very high mutation frequency. One would expect that only an insertion, deletion, or rearrangement, mediated by an insertion element could give a sufficiently high frequency.

Table 3.2 Yield of Tmp^R,Ts transductants in the transductional cross ED419 (dnaA46,Tn7) x C600 or R2D2

donor	recipient	no. of Tmp^R clones tested	no. of Tmp^R clones temperature-sensitive
ED419Tn7	C600	50	32
clone1	R2D2	50	20
ED419Tn7	C600	50	25
clone2	R2D2	50	23

Fig 3.2 shows the result of hybridisation between sidA DNA and genomic digests of C600 DNA. This demonstrates that the restriction patterns of the C600 chromosomal sidA region and the cloned sidA fragment are the same. Thus, so far as can be determined, the sidA fragment is wild-type DNA.

3.6 Effect of molecular environment on sidA expression

A possible explanation of the expression of the sidA phenotype in λsidA1 lysogens is that the sidA gene is linked to an external promoter in its new molecular environment and thus its expression is stimulated.

Consideration of Fig. 3.3 indicates that sidA might be transcribed from the P_i promoter which gives weak constitutive expression reading leftward through att, although this could only occur if the phage were lysogenised by homology, since the attachment site lies between P_i and the sidA insert. The lac promoter is deleted when the central EcoR1 fragment of λNM616 is replaced.

To further investigate the effect of molecular environment on sidA expression, a derivative of λsidA1 in which the chromosomal insert had been inverted was prepared: 1ug of λsidA1 was restricted with EcoR1 and the ligated DNA was packaged *in vitro*. Due probably to excessive dilution of the DNA, only 12 plaques were isolated, of which 3 were very small and proved impossible to propagate. These latter were probably the religated arms of λNM616 which are of insufficient length (37.5 kb) to be efficiently packaged. The remaining nine phage plaques were amplified by a single cycle lysate on SM32, then DNA was prepared by the rapid method described in 3.3 and digested with EcoR1 to check that the right insert had been obtained and with HindIII to determine the orientation. The sidA insert has a HindIII site very close to one end, which allows ready discrimination of the two insert orientations.

One of the inversion derivatives, called λsidA2, was further purified. When this phage was spotted onto ED419 (dnaA46) growth in the spot at

Figure 3.2

A : Hybridisation of sidA to chromosomal DNA

Track	DNA	Enzymes
1	pND5	HpaI
2	W3110 genomic	HpaI
3	C600 genomic	HpaI
4	pND5	HpaI,EcoR1
5	W3110 genomic	HpaI,EcoR1
6	C600 genomic	HpaI,EcoR1
7	pND5	EcoR1
8	W3110 genomic	EcoR1
9	C600 genomic	EcoR1

The probe was pND5. The additional band of approximately 4kb that lights up in HpaI and HpaI/EcoR1 digests of C600 genomic DNA (tracks 3 and 6) is presumably a partial digestion product, possibly consisting of the contiguous 2.3kb and 1.65kb Hpa1 fragments (see fig 3.3). The 6.0kb band is pBR325. HpaI cleaves pND5 very close to each end of the sidA insert and thus the pBR325/sidA junction fragment (track 1) has a mobility only slightly less than that of pBR325 (tracks 4, 7)

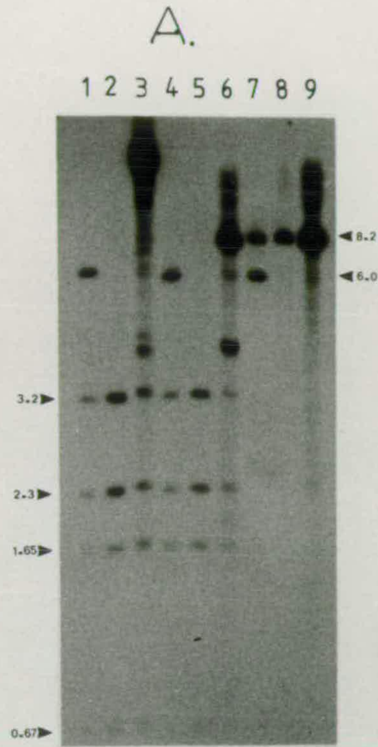
B1, B2 : Absence of homology between sidA and dnaA or sdaE

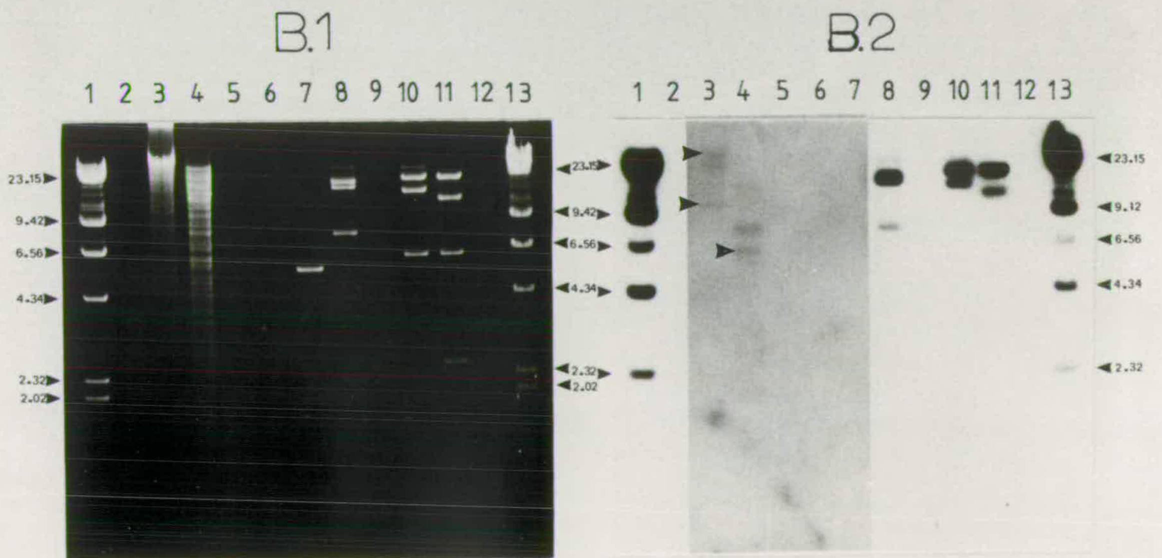
track	DNA	Enzyme
1	λCI857	HindIII
3	C600 genomic	HindIII
4	C600 genomic	EcoR1
7	pSP6	HindIII
8	λsidA1	EcoR1
10	λ425	HindIII
11	λ425	HindIII,XhoI
13	λCI857	HindIII

The faint 2.5kb sdaE band of pSP6 in track 7 is just discernible on the original of this figure, but is not visible in this reproduction. The dnaA gene is carried by the 2.4kb HindIII/XhoI fragment in track 11

The gel was transferred to nitrocellulose and probed with λsidA1. The inset (tracks 3 - 7) is of a 50 day exposure. The rest of the autoradiogram was exposed for 36hrs. The 8.2kb sidA EcoR1 band is clearly visible in track 4. Bands judged to be due to λ homology by comparison with the results of Kaiser & Murray (1979) are marked with an arrow.

Fig. 3.2





$40^{\circ}C$ indicated that this phage was able to suppress dnaA. Thus, barring the existence of two unidentified promoters reading into the central region of λ during lysogeny, transcription of sidA from an external promoter is eliminated as an explanation for its suppressing activity.

3.7 Recloning of sidA in high copy number

The 8.2kb sidA insert was also recloned into the EcoR1 site in the cat (Chloramphenicol resistance, Cm^R) gene of pBR325. Due probably to a cold sensitivity phenotype which sidA plasmids confer on certain strains, including the chosen transformation host C600 (see sections 3.9, 3.11 & 3.12), of 15 Tet^R,Cm^S transformants only 1 contained an 8.2kb insert. The remainder appeared to be the result of deletions of the recombinant plasmid, since they contained (for the most part) only one EcoR1 site and were of a slightly higher molecular weight than the vector.

This plasmid, pND5, also suppressed dnaA. When ED419 was transformed with pND5, it was able to grow not only at $40^{\circ}C$ (the maximum growth temperature for λsidA1 lysogens), but also at $42^{\circ}C$. Two factors may account for this increased efficiency of suppression. The first is the high copy number of the plasmid and the second is the fact that two promoters (the cat promoter and the tet anti-promoter) read into the EcoR1 site of pBR325, one from each direction (Fig. 3.3).

pND5 was used to prepare a plasmid in which the insert was inverted. This construction proved difficult; in a typical experiment 0.5µg of pND5 was restricted with EcoR1 and religated in a volume of 10µl. Of 10 Tet^R,Cm^S transformants screened, 5 had the insert orientation of the parent plasmid and the other 5 were "monsters" - plasmids which had the restriction patterns neither of the vector, nor the parent plasmid, nor the anticipated inversion product. Some 70Cm^s clones were screened before a plasmid, pND6, with the HindIII and EcoR1 restriction patterns expected for the inversion plasmid was obtained. Fig. 3.4 shows this plasmid, together with some of the "monsters" obtained in

Figure 3.3

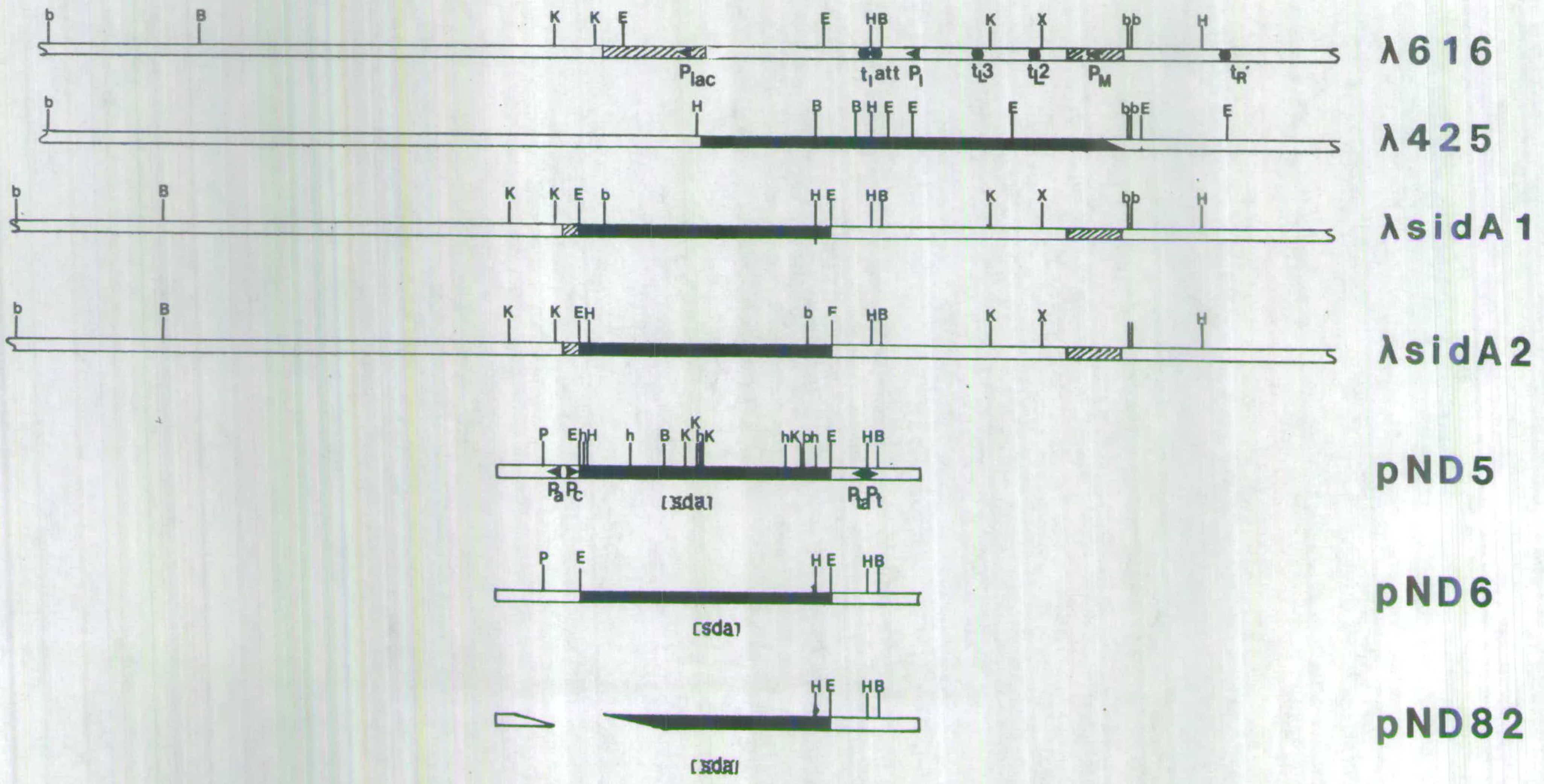


Figure 3.3 : Molecular maps of sidA episomes.

The positions of promoters active during λ lysogeny (◄), terminators, and the λ attachment site are indicated.

λ promoters

P_m Promoter for repressor maintenance (cI promoter)

P_i Promoter for int

Plasmid Promoters

P_a Promoter for the bla (amp^R) gene

P_c Promoter for the cat (cm^R) gene

P_t Promoter for the tet gene

P_{ta} The tet anti-promoter

The position of the region identified by Takeda and Hirota as essential for dnaA suppression is shown by a boxed "sda".

Restriction targets :

b = BglII
B = BamH1
E = EcoR1
h = HpaI
H = HindIII
K = KpnI
P = PstI
X = XhoI

Restriction sites in λ 616 DNA are from Williams and Blattner,1981 and the positions of promoters are from Daniels et al.,1983.

Solid lines: chromosomal DNA;
Open lines: Vector DNA;
Hatched lines: DNA from the lac5 and imm21 substitutions.

The right hand end of λ425 was generated by an in vivo extension. The ends of the slanted boundary between chromosomal and phage DNA indicate the uncertainty in the positions of the deletion/insertion endpoint.

the same experiment.

Strains carrying pND6 form very slow-growing colonies and the strain carrying it grew slowly in liquid media. For this reason, large scale preparation of this plasmid proved impossible, since in the absence of selection for temperature resistance the cultures were overgrown by cells carrying deletion derivatives which did not suppress dnaA. Three such derivative plasmids obtained in such a fashion are shown in Fig. 3.4 B.

Like pND5, pND6 is able to protect ED419 up to 42°C, so this plasmid likewise expresses sidA strongly as would be expected from its high copy number.

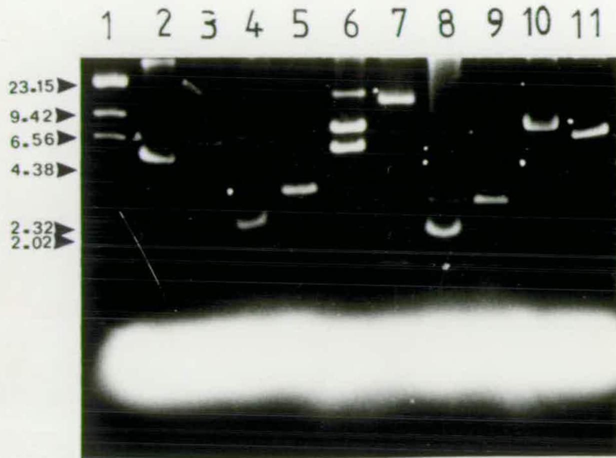
The fact that sidA plasmids suppress dnaA more efficiently than the phage indicates that the suppression is trans-acting, and therefore presumably due to a diffusible product. This eliminates one possible route of suppression of dnaA: integrative suppression, where chromosome replication is driven by an integrated dnaA-independent origin of replication. Although it is possible to produce integrative suppression by high copy number plasmids (Yamaguchi & Tomizawa,1980), if sidA contained an origin of replication, then actual stable integration into the chromosome by λ lysogeny would be a more efficient means of providing suppression. Furthermore, studies on a suppressor that is identical to sidA (see 3.8) by Takeda and Hirota (1982) show that high copy number plasmids carrying this gene can suppress dnaA in RecA strains where the suppressor can act only in trans.

Fig. 3.3 shows the four sidA recombinants: λsidA1, λsidA2, pND5, and pND6. A map of the deletion derivative pND82 is also shown. This plasmid does not suppress dnaA, so an approximate estimate of the position of the sidA locus can be made, based on the following reasoning.

The size of pND82 is estimated as 10.1kb, and that of its parent plasmid, pND6 is 14.2kb. Thus approximately 4kb of DNA has been deleted from pND6 to produce pND82.

Figure 3.4

A



B

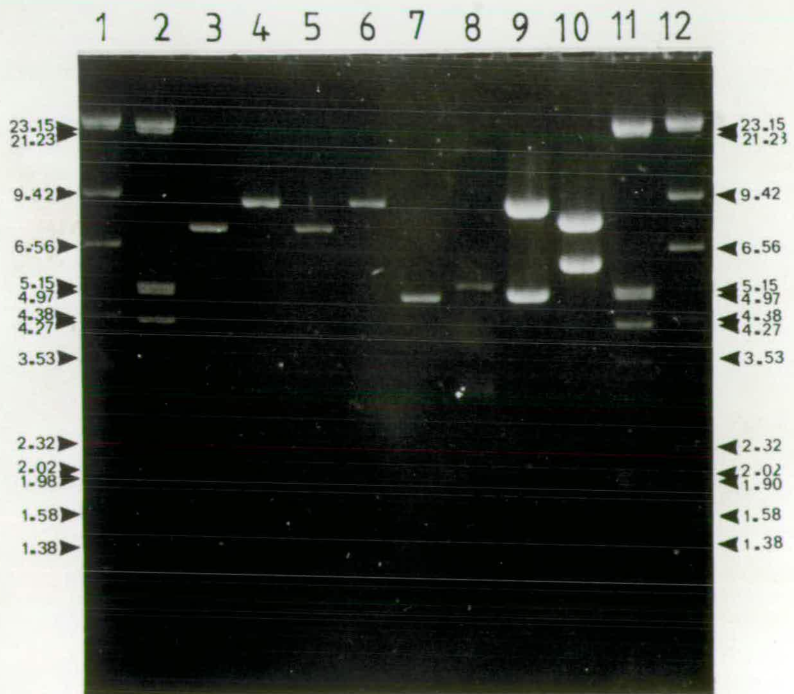


Figure 3.4

A: pND6 digested with EcoR1 (track6) and HindIII (track7). The λcI857 standard is in track 1.

4 deletion plasmids, digested with EcoR1 (tracks 2,4,8,10) and HindIII (tracks 3,5,7,11) are also shown. The plasmid in tracks 10 and 11 is apparently similar to pND82 (see B).

B: Plasmids derived from pND6 : pND82 (tracks 3 + 4), pND84 (tracks 5 + 6) and pND35 (tracks 7 + 8). pND82 and pND84 are apparently identical. The 1.5kb fragment of pND82 and pND84 stretching from the HindIII site in sidA through the promoter-distal end of cat to the tet promoter is visible in track 3 but too faint to discern in track 5. This fragment is characteristic of pND6 derivatives (see A, tracks 7 and 11)

track	DNA	Enzyme
1	λcI857	HindIII
2	λcI857	HindIII+EcoR1
3	pND82	HindIII
4	pND82	EcoR1
5	pND84	HindIII
6	pND84	EcoR1
7	pND35	HindIII
8	pND35	EcoR1
9	pND5	HindIII
10	pND5	EcoR1
11	λcI857	HindIII+EcoR1
12	λcI857	HindIII

This deletion removes the left-hand EcoR1 site of pND6 as drawn in Fig. 3.3, since the plasmid has only one EcoR1 site, and retains the HindIII restriction pattern characteristic of the right hand end of pND6.

The deletion also removes part of the amp gene, since pND82 does not confer ampicillin resistance on its host. Thus, a minimum of 670bp (the distance between the start of the amp gene and the EcoR1 site in pBR325) has been deleted from the vector DNA and therefore a maximum of 3.3kb has been deleted from the sidA insert.

Since the plasmid is able to replicate, its origin of replication must be intact. The origin of replication of pBR325 is 2.2kb from the EcoR1 site and this sets the lower limit on the amount of DNA deleted from the sidA insert: 4 - 2.2 = 1.8kb.

Thus, some of the DNA lying between 1.8kb and 3.7kb from the left hand EcoR1 site of pND6 is essential for expression of the sidA phenotype.

3.8. sidA is identical to a previously identified suppressor.

During the course of this work, Takeda and Hirota (1982) published the restriction map and some other details of a plasmid, pYT47, which also suppressed the dnaA46 mutation and had an 8.2kb insert. To check whether sidA and the pYT47 insert were the same, a restriction map of the sidA fragment was prepared by the analysis of double digests with EcoR1, HindIII, BglII, BamHI, KpnI, HpaI, and XhoI. This confirmed that the two fragments are indeed identical (or at least very closely similar).

Of the remaining multicopy suppressor fragments shown in Fig. 1.3, only pSP6 has a restriction map that could overlap sidA, since both fragments have a short HindIII-EcoR1 fragment at one end. To investigate this possibility, pSP6 was probed with λsidA1 (Fig 3.2). No hybridisation was detected.

3.9 Physiology of SidA Strains

Table 3.3 shows growth rates for strains carrying pND5 and λ*sidA*.

Representative growth curves for *dnaA*, *dnaA*+, and *dnaA*,λ*sidA1* strains are shown in Figs 3.5 and 3.6.

Although in some cases (for instance ND61) a wide spread of growth rates is observed (an effect which may be due to differences in incubation conditions of starter cultures (see 3.12)), SidA strains generally grow more slowly than isogenic controls, an effect that is more pronounced at 30°C and in strains carrying the *dnaA46* mutation, where it is possible to observe depressed growth rates in strains carrying pND5 (ND81) or λ*sidA* (ND61)

The growth defect is particularly severe for strains carrying pND6. Cultures of strains carrying pND6 achieved only a moderate density (O.D. appr. 0.3) after overnight growth in broth at 37°C, and microscopic examination of the cells revealed extensive "blebbing" (a sign of incipient lysis). This effect is also seen in ampicillin-sensitive cells growing in the presence of ampicillin, but since the selective antibiotic used was tetracycline, the suggestion that this effect is due simply to plasmid instability and the effect of antibiotic on plasmid free cells can be rejected. The plasmid pND6 is, however, very unstable as has been noted above (3.7), and as a result it was not possible to quantify growth rates for strains carrying this plasmid.

The depression of the growth rate of C600 by pND5 shown in Table 3.3 is probably only part of the maximum effect, since when pND5 transformants of C600 were initially sought they were found to grow very slowly, taking some 3 days to appear at 30°C. However, subculturing seemed to select for derivatives with near normal growth rates. Since ND80 had been maintained in culture for some time it is probably such an "adapted" variant.

Table 3.3 Growth rates of SidA strains

Strain	Background	Relevant genotype	Temperature 30°C	37°C	40°C	42°C
ED419		dnaA46	41.4+/-2.4	25	ND	27
ND61	ED419	dnaA46 λsidA1	60.3+/-15.2	ND	35.3+/-1.9	ND
ND70	ED419	dnaA+	46.4+/-7.0	ND	30.0+/-5.25	ND
ND81	ED419	dnaA46 pND5	78	39	ND	50
ND68	ED419	dnaA+ λsidA1	45-64.5	ND	24.5	ND
ND69	ED419	dnaA+ λ616	45-50.3	ND	ND	ND
ND13		polA	ND	30	ND	ND
ND91	ND13	polA pND5	ND	41	ND	ND
C600			54	33	ND	30
ND80	C600	pND5	61	33	ND	30

Figures are doubling time in minutes in LB, determined by periodic measurement of A_{540} of exponentially growing cultures. The growth rate of ED419 at 42°C is the initial growth rate after shift to 42°C.

ND: not determined.

Results based on 3 or more estimates are given standard errors, those based on 2 estimates are given as a range, and single figures represent single estimates.

This "adaptation" effect was more pronounced in the dnaA46 strain ED419, although, in this case, slow growth seemed to be a direct consequence of the presence of the dnaA mutation, since an isogenic dnaA+ strain carrying pND5 grew normally. When pND5 transformants of ED419 were selected at 30°C, these formed small colonies after 3 days, whereas pND5 transformants of ND70 (DnaA+) and pBR325 transformants of both strains formed normal sized colonies after 24 hours. When the slow-growing colonies were subcultured at 30°C they were found to give rise to large colony forming variants. Therefore, "unadapted" ED419,pND5 are cold sensitive, having a depressed growth rate relative to wild-type and dnaA strains at 30°C. The growth rate of ED419,pND5 is also depressed relative to the wild type at 42°C. However, at 42°C, cell morphology of ED419,pND5 is normal, while at 30°C the cells are noticeably filamentous. Adapted colonies have a normal cell morphology, but retain their temperature resistance. Cold sensitivity appears to be the result of an interaction between sidA and dnaA46, since the presence of both is required for cold sensitivity to be observed. A similar effect has been observed in strains where dnaA is combined with a das suppressor mutation (Atlung,1981), in strains that overproduce the dnaA46 mutant gene product (von Meyenburg et al.,1985 in press), and in strains diploid for dnaA+ and certain dnaA(Ts) alleles (Hansen et al.,1984).

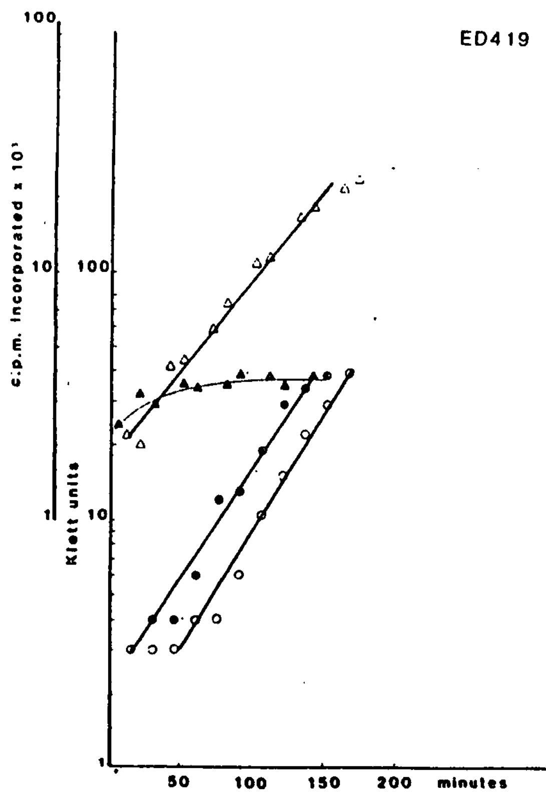
Since the slow growth rates of C600,pND5 and ED419,pND5 appear to be (in part at least) due to different causes, I refer to them as "slow growth rate" (C600) and "cold sensitive filamentation" (ED419). The cause of adaptation in either strain is not known at present. Reversion of ED419 to dnaA+ is a likely explanation for adaptation of this strain, but this clearly could not be the cause of adaptation in C600, which is already dnaA+.

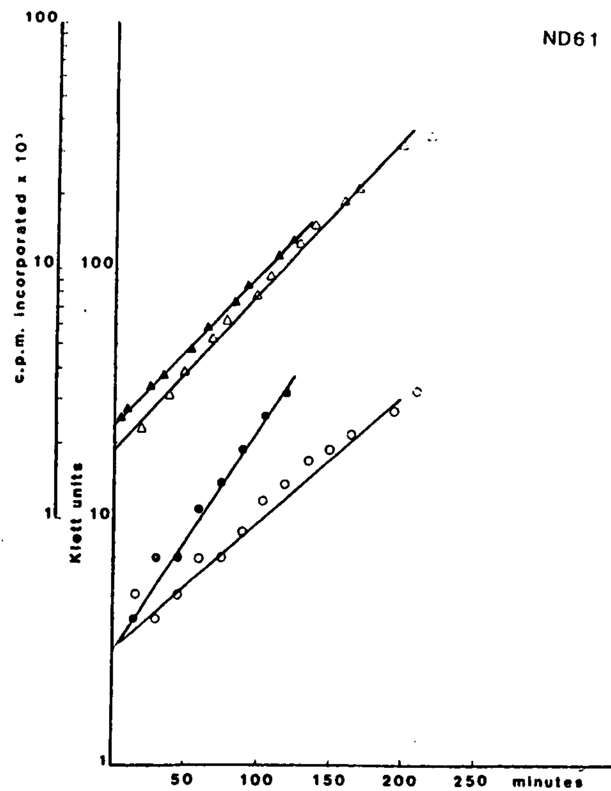
Fig. 3.5 shows growth and DNA synthesis of ND61 (dnaA46,λsidA1) and ED419 (dnaA46) after a shift from 30°C to 40°C. It is conspicuous that while the doubling time of ND61 decreases from 52-60 mins at 30°C to 37' at 40°C (a 1.4-1.6 fold decrease), the rate of DNA synthesis remains unchanged. As a result, while cell mass increases 8X, DNA increases only 4X over the 150 mins of the experiment. Over a corresponding period

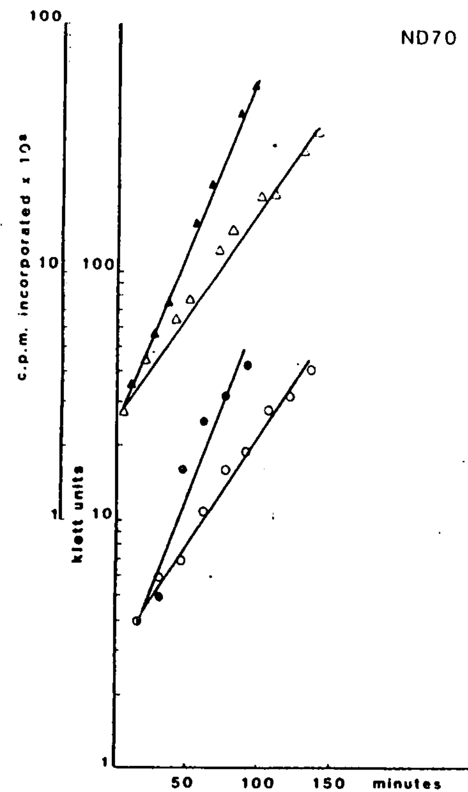
Figure 3.5

Increase in cell density (circles) and DNA synthesis (triangles) of ED419 (dnaA46), ND70 (dnaA+) and ND61 (dnaA46,λsidA) at 40°C (filled symbols) and 30°C (open symbols). Cultures were prelabelled for 3 generations in LB + 200µg/ml uridine + 25µCi/ml ^{3}H-thymidine + 6.5µg/ml cold thymidine at 30°C before dilution into the same medium at 30°C and 40°C. 50µl samples were collected onto Whatman 3MM paper, precipitated in trichloroacetic acid, washed and dried. Radioactivity measured by liquid scintillation counting.

Figure 3.5







at 30°C (250 mins) both DNA and mass increase 8X in parallel. This is reflected in a decline in the ratio of DNA to cell mass which is initially similar to, or slightly less than that of a dnaA+ control, and declines in the course of the experiment (see Table 3.4).

ND70 (dnaA+) and ED419 (dnaA46) behave as expected: ND70 shows parallel growth and DNA synthesis at 30°C and 40°C, while ED419 shows the "slow stop" of DNA synthesis characteristic of dnaA strains.

Apparently, therefore, DNA synthesis lags behind cell growth after a shift from 30°C to 40°C in ND61. This is necessarily a transient effect, but it is not clear whether the rate of DNA synthesis "catches up" or whether the growth rate slows down to the DNA synthesis rate. In any case, the average DNA content of the cells decreases. This could be compensated for by an increase in cell mass or by production of enucleate cells:

1) Increase in cell volume. Fig. 3.6 shows the effect of temperature on growth, cell division and median cell volume for the strains listed in Table 3.4. ND61 does not appear to behave any differently from the dnaA+ strain, so there is no noticeable effect of the dnaA,λsidA combination on cell division. In particular, there is no increase in cell volume on shifting from 30°C to 40°C.

2) Production of enucleate cells. If enucleate cells are produced on shift from 30°C to 40°C, one expects to see a drop in the viability of the cultures. To test this, cultures growing exponentially at 30°C and 40°C were diluted and plated to single colonies. The viable count thus obtained was compared with the cell density as estimated from the optical density of the culture. The results are shown in Table 3.5.

The reduced viability of cultures grown at 40°C suggests that production of enucleate cells is a possible explanation of the discrepancy between growth rate and rate of DNA synthesis in suppressed λsidA1

Table 3.4 : DNA/mass ratios in DnaA+, DnaA- and DnaA-, λsidA strains

	ED419		ND61		ND70	
	30° C	40° C	30° C	40° C	30° C	40° C
Time after dilution (mins.)						
50	1.33	0.72	0.73	0.59	1.0	1.0
100	1.08	0.23	0.82	0.41	1.2	1.1
150	0.77		0.94			

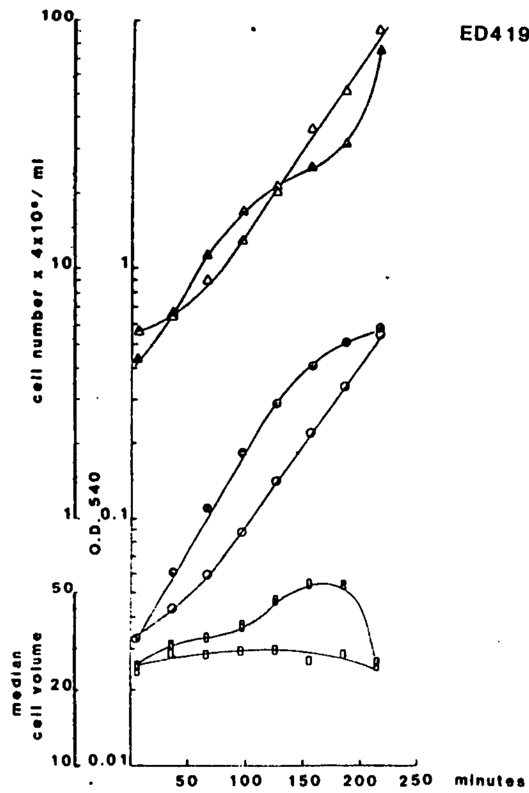
Results are calculated from the data presented in Fig. 3.6. The ratio is in units of cpm/50μl/Klett unit x 10^{-3}. Values for both radioactive counts and cell density are unfortunately too small to allow accurate estimation of the ratio at T=0.

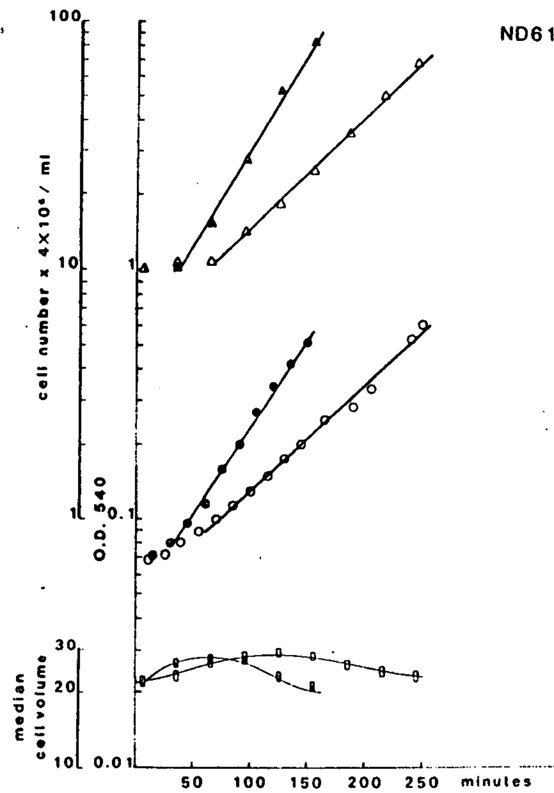
Figure 3.6 : Cell division in ND61 (dnaA46,λsidA), ND70 (dnaA+), and ED419 (dnaA46)

Cultures growing exponentially in L-broth at 30°C were diluted into the same medium at 30°C (open symbols) and 40°C (filled symbols). Cell density (circles) was followed, and periodic samples were removed into 20% formaldehyde for determination of cell number (triangles) and cell size (rectangles).

The complex behaviour of ED419, which seems to involve an initial inhibition of cell division about 100 minutes after shift to 40°C (or 60 minutes after cessation of DNA synthesis) followed by a resumption of cell division may reflect an initial inhibition of septation by centrally placed nucleoids in cells that would otherwise divide centrally. When division is resumed, it is assymetrical, and results in the formation of enucleate cells (data not shown).

Figure 3.6





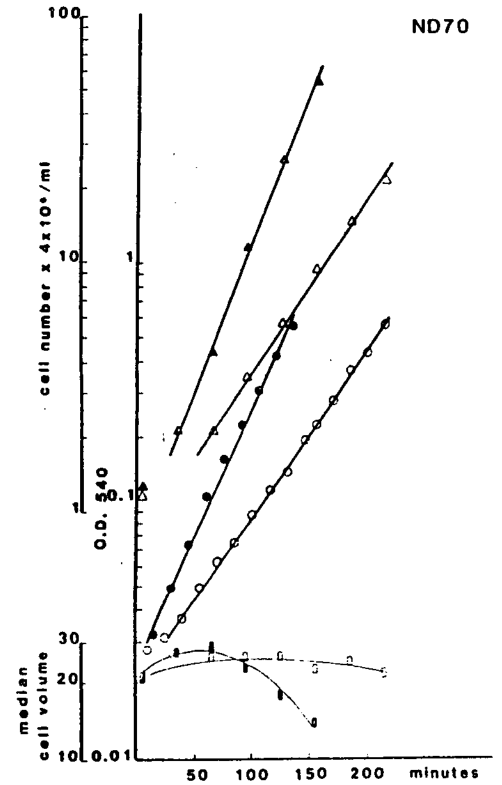


Table 3.5 Effect of temperature on cell viability of ND61

Strain/Growth temperature	Bacteria/ml x 10^{-8}		
	cell density (1)	viable counts (cfu)	ratio viable counts: cell density
ED419/30°C	4.4	6.0	1.36
ND61 /30°C	3.5	4.0	1.14
ND61 /30°C	2.5	3.1	1.34
ND61 /30°C	2.5	3.5	1.4
ND61 /30°C	1.8	1.3	0.72
			mean = 1.13+/-0.25
ND61 /40°C	1.2	0.51	0.42
ND61 /40°C	1.5	0.86	0.57
ND61 /40°C	2.32	1.3	0.56
ND61 /40°C	0.68	0.16	0.24
			mean = 0.45+/-0.15

(1) estimated on the basis that an OD of 0.5 is equivalent to a cell density of $2x10^{8}$/ml for broth-grown cells.

lysogens.

3.10 The effect of sidA on nalidixic acid resistance

During experiments to map the chromosomal *sidA* locus by Hfr conduction (detailed in 3.13) it was noticed that Tet^R exconjugants were obtained when mating was interrupted by vortexing and the addition of streptomycin, but not when 100ug/ml nalidixic acid was added to the mating mixture to effect interruption. The low levels of pBR325 transfer did not seem to be affected by nalidixic acid.

It seems, therefore, either that nalidixic acid is preventing establishment of pND5 or that pND5 renders the recipient strain, MM7, sensitive to nalidixic acid. To investigate this further, pND5 transformants of MM7, and λ*sidA1* lysogens of CND18 (a spontaneous Nal^R derivative of C600K-), were tested for nalidixic acid sensitivity. Table 3.6 shows that pND5 confers nalidixic acid sensitivity on MM7, but that λ*sidA1* does not affect the Nal^R phenotype of CND18.

Table 3.6: Effect of SidA on nalidixic acid resistance

Strain	Growth on 20µg/ml Nal
CND18	+
CND18 λ*sidA1*	+
MM7 pBR325	+
MM7 pND5	-

Nalidixic acid does not completely inhibit growth of MM7pND5, but both the size and number of colonies is drastically reduced.

It would seem either that pND5 cannot be maintained in the presence of naladixic acid, even in a Nal^R host (due, for instance, to extreme genetic instability of the sidA DNA in pBR325, but not in λ), or that sidA, or some other function on the sidA DNA suppresses Nal^R.

3.11 The range of mutations suppressed by λsidA

A study of the range of mutations suppressed by sidA should serve to distinguish between the possibilities of its acting by interacting directly or indirectly with the dnaA gene or its product (in which case, some alleles of dnaA would not be suppressed), or by bypassing the dnaA function entirely (in which case all dnaA alleles should be suppressed). In fact the results obtained so far are too complex to allow this simple distinction to be made.

Initiation mutations in dnaB, dnaC, and dnaP, as well as a number of dnaA alleles have been tested for suppressibiliy by sidA. Three tests have been applied:

1) Spot testing - A lysate of λsidA was spotted onto a lawn of the mutant strain and plates were incubated at 30°C and 40°C. Growth in the spot at 40°C was regarded as a positive result

2) Isolation of lysogens at 30°C and testing for temperature resistance by streaking out at 30°C and 40°C

3) Determination of viable counts at 30°C and 40°C for the same lysogens examined by method 2.

4) Transformation with plasmid pND5 and plating transformants at 30°C and 42°C

The results for these tests are summarised in Table 3.7.

A number of points emerge from these tests:

1) Of the mutants tested, only dnaA mutants are detectably suppressed by λsidA1. (Note that dnaC1 is an elongation-defective mutation). Within this sample, sidA is a specific suppressor for dnaA.

2) Not all alleles of dnaA are equally well suppressed by λsidA1. Suppression of dnaA508 and dnaA204 can be detected by plating out or streaking lysogens, but not in a spot test. Since selection and subsequent testing of lysogens for temperature resistance involved many generations of growth at 30°C, it may be that the positive results obtained by this method and not by spot testing may reflect a delay in the establishment of the suppressed phenotype in dnaA204 and dnaA508 backgrounds.

3) Some aspects of the genetic background also appear to affect suppression of dnaA. Although the dnaA5 mutation is suppressed in both JW397 and JW398, suppressed colonies of JW397 were much smaller than those of JW398. These strains are isogenic except for the presence of the zib501::Tn10 insertion in JW397, which is very closely linked to dnaA. It would appear that this insertion in some way interferes with the suppression process, either by reducing the efficiency of dnaA suppression, or by augmenting the growth rate depression that is seen in some λsidA1 lysogens (see below). Alternatively, since JW398 was derived from JW397 by curing the zib501::Tn10 insertion (J. Wechsler, Pers. Comm.), it is possible that a deletion of DNA in the dnaA region occurred during the curing process, and this relieves a slow growth phenotype caused by interaction between sidA and dnaA5. A similar explanation might account for the observation that while the dnaA204 mutation in WM1152 is suppressed by λsidA1, the same mutation in JW393 is not suppressed by pND5. JW393pND5

Table 3.7. Range of alleles suppressed by λsidA1

mutation	strain	spot test(1)	streak(2)	viable counts(3)	plasmid(4)
dnaA5	JW397	+	NT	NT	NT
dnaA5	JW398	+	NT	NT	NT
dnaA46	ED419	+	+	+	+
dnaA46	WM1026*	+	+	+	NT
dnaA46	ND65	+	NT	NT	NT
dnaA167	WM1029*	NT	-	-	NT
dnaA177	E177	+	+	NT	-
dnaA204	WM1152	-	+	+	NT
dnaA204	JW393	NT	NT	NT	-
dnaA508	WM1032*	-	+	NT	NT
dnaA508	JW402	-	NT	NT	NT
dnaC2	PC2 **	-	NT	NT	-
dnaC1	PC1 **	-	NT	NT	-
dnaP18	CND18	-	NT	NT	NT
dnaB252	RS162	-	NT	NT	NT

NT: Not Tested

(*),(**): Strains marked thus are isogenic.

Notes

Spot test: all strains tested were sensitive to λsidA1.

Streak test: a positive result was recorded if any of the twelve lysogens tested grew better at 40°C than non-lysogenic controls.

Viable counts: a positive result was recorded if any of the lysogens tested produced at least half as many colonies at 40°C as at 30°C.

WM1029: this strain is viable at 40°C, therefore tests for temperature resistance were conducted at 42°C.

transformants form slow-growing, flattened colonies indicative of low viability. Evidently, therefore, either the JW393 genetic background, or the dnaA204 mutation itself interacts deleteriously with pND5. JW393 is a B/r derivative and is not isogenic with JW397 or JW398.

4) E177 is suppressed by λsidA, but not by pND5. In view of the fact that at 30°C, E177pND5 transformants were neither scarce nor slow-growing, it is difficult to account for this observation. Plating of transformants at 30°C, 37°C, 40°C and 42°C showed that E177pND5 is more temperature-sensitive than E177pBR325; E177pBR325 is able to form colonies at 37°C, while E177pND5 is unable to form colonies at this temperature. Should this result prove reproducible, it would suggest that moderate overexpression of sidA suppresses temperature-sensitivity in E177, while a greater degree of overexpression either compounds the temperature-sensitive phenotype of E177, or engenders an independent temperature-sensitive phenotype.

It is apparent that the allele specificity of sidA suppression is complicated. Effects range through temperature resistance without cold sensitivity ("adapted" ED419pND5), temperature resistance with cold sensitivity (ED419pND5, WM1026λsidA1, see below), enhanced temperature sensitivity (E177pND5) and apparent loss of viability at all temperatures (JW393pND5). In many of the λsidA1 lysogens studied, there is no apparent effect on the phenotype of the host at all. What effect is evinced by sidA depends on copy number, molecular environment, and genetic background, as well as the dnaA allele tested.

For this reason, it is not possible to say whether sidA causes bypass of dnaA function (as occurs in rnh mutants constitutive for stable DNA replication (Kogoma & von Meyenburg,1983)) or whether the participation of a partially active dnaA product is needed for suppression to occur. One approach to solving this problem, which is important to our understanding of the mechanism of sidA suppression, would be to attempt to separate the effects of the various dnaA alleles from the

effect of genetic background. This would be time consuming but instructive in terms of the other phenotypic effects of sidA since host adaptation and differences of suppression in various strains presumably reflect the interaction of other genes with sidA. Alternatively, the question could be more simply answered by attempting to introduce the null allele dnaA850::Tn10 into a strain in which sidA is known to be active (such as a suppressed lysogen of WM1026; see below).

The results with dnaA167 (WM1029) do suggest that sidA suppression is allele-specific, since while the isogenic dnaA508 and dnaA46 strains WM1032 and WM1026 can be suppressed by λsidA, WM1029 is not suppressed. It is however necessary to qualify this since:

a) dnaA167 is only temperature-sensitive at 42°C, and none of the alleles tested are suppressed for growth at 42°C by λsidA1.

b) Results presented in 3.13 showing that two kinds of λsidA1 lysogens are formed, and that only one of these gives suppression. It is conceivable that the dnaA167 background is in some way inimical to the formation of the suppressing type of lysogen.

3.12 λsidA1 can form two phenotypically distinguishable types of lysogen.

Even in strains such as WM1026 (dnaA46), ED419 (dnaA46) and E177 (dnaA177) which are clearly readily suppressed by λsidA1, not all lysogens are temperature-resistant. To further investigate this, 20 lysogens of WM1026 were isolated without exposure to restrictive temperature. Only 3 out of a total of 20 tested were found to be temperature-resistant. This was not due to genetic heterogeneity of the lysogenising phage, since all the lysogens were able to produce suppressing phage.

Suppressed and non-suppressed lysogens differed in their growth rates at 30°C as well as their temperature resistance. During the process of preparing phage from these strains by UV induction, it was noticed that suppressed and non-suppressed lysogens also differed in

their response to UV irradiation. Their response to UV irradiation is shown in Fig. 3.7 and the differences in properties are summarised in Table 3.8.

Suppressed lysogens grow more slowly than non-suppressed lysogens, show a more dramatic effect of UV irradiation on their growth rate and lyse less on UV induction of their lysogenising phage, Surprisingly, therefore, higher yields of phage are produced by suppressed lysogens than by non-suppressed lysogens.

In their initial growth rate depression in response to UV irradiation, the suppressed lysogens resemble lysogens of λ425. This phage is deleted for the att-int region. It is thus unable to integrate by the efficient attλ-specific integration system and must instead integrate by recA dependent recombination at dnaA.

Two explanations for the similarity between λ425 lysogens and suppressed λsidA1 lysogens spring to mind. The first is that both λ425 and λsidA1 lysogens have excessive activity of dnaA protein before or after induction, and that this causes slow growth in response to UV irradiation. The second is that suppressing λsidA1 prophage are, like λ425, integrated by homology and that homologously integrated phage excise from the chromosome less efficiently than int-mediated prophage, so that replication in situ amplifies the neighbouring chromosomal genes to a deleterious degree. These are not mutually exclusive explanations.

The possibility that excessive dnaA activity causes slow growth in UV-irradiated cells is interesting in the light of the fact that at least one of the genes involved in the repair of UV-damaged DNA, uvrA, has a dnaA binding site upstream of its promoter (Fuller et al.,1984). If, however, amplification of sidA and consequent excessive dnaA activity is responsible for slow growth after UV irradiation, it is hard to understand why non-suppressing prophage, which would also be expected to overexpress sidA after induction, give a normal response to UV irradiation. It will be interesting to investigate the effect of UV irradiation on strains overexpressing dnaA and sidA but free of inducible

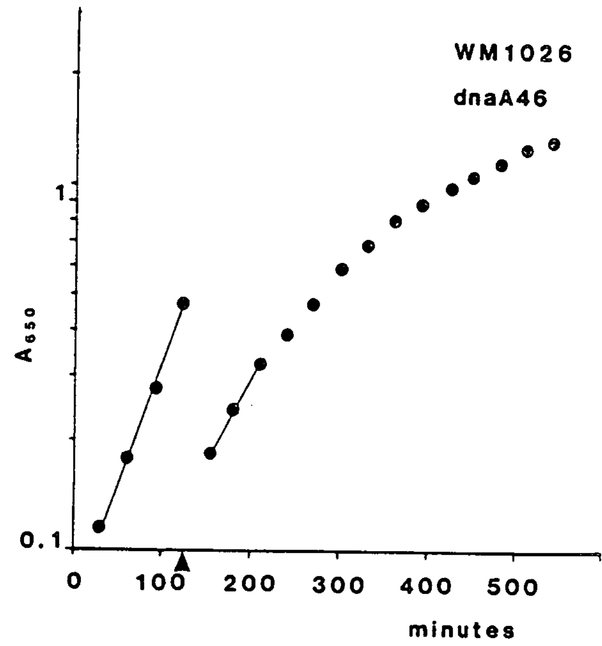
Figure 3.7: Growth and response to ultraviolet irradiation in suppressed and non-suppressed λsidA1 lysogens of WM1026

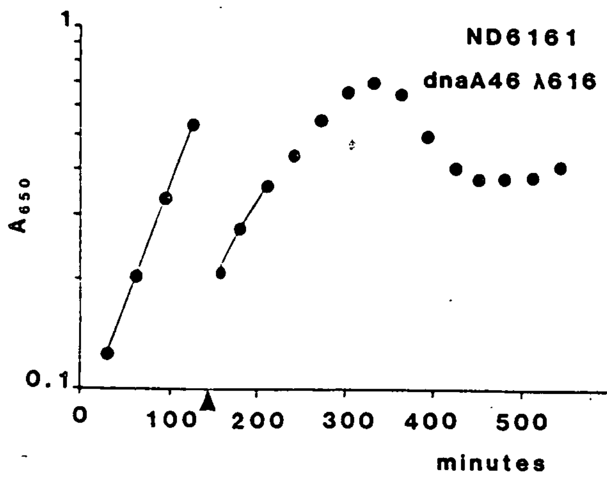
Overnight cultures at 40°C (ND6127, ND6251) or 30°C (ND6161, ND6138, WM1026) were subcultured in LB at 30°C. Optical density was measured periodically until the cultures reached an O.D. of 0.5, when the cells were harvested, resuspended in 1/2 vol 10^{-2}M $MgSO_4$ and exposed to 400 ergs/mm^2 of UV light from a germicidal lamp. They were then diluted fourfold into prewarmed LB and the OD was followed until it ceased to decrease, when the cultures were chloroformed and treated as usual for phage lysates.

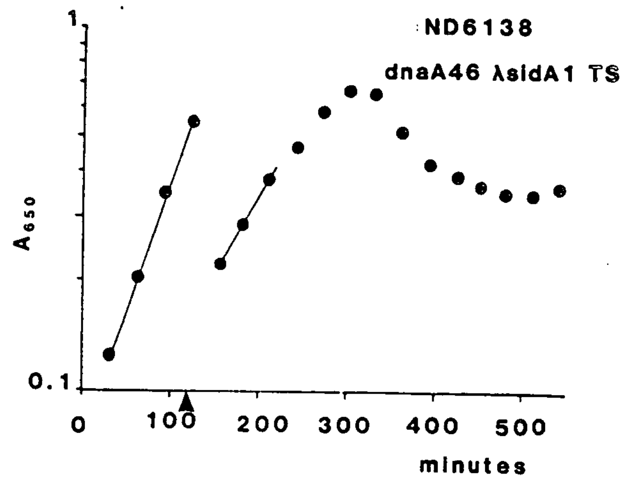
Growth rate after UV irradiation was determined by taking the initial growth rate for the period over which growth was exponential. This is indicated by the line drawn through points after irradiation.

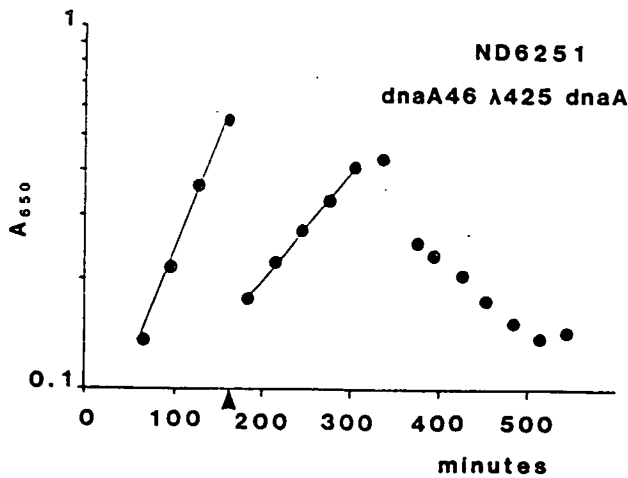
Time of UV irradiation is indicated by an arrow.

Figure 3.7









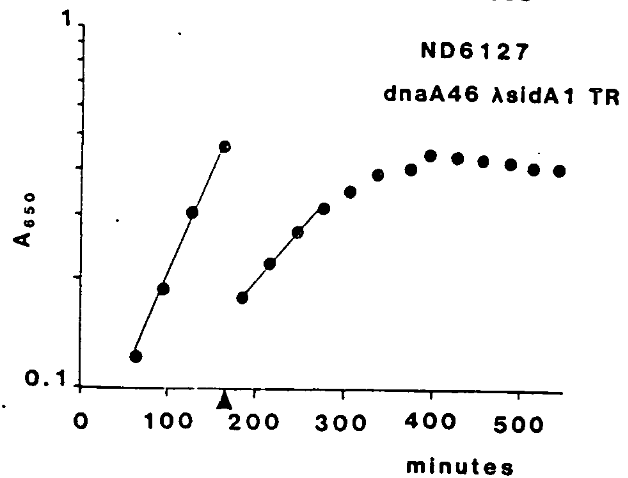


Table 3.8 Properties of suppressed and non-suppressed lysogens of λsidA1

strain	description	growth rate (30°C)	growth rate after UV irradiation	production of suppressing or complementing phage
WM1026	dnaA46,λ-	45'	64'	-
ND6161	WM1026,λNM616	43'	65'	-
ND6251	WM1026,λ425dnaA+	48'	100'	+
ND6101	WM1026,λsidA1,TR	52'	105'	+
ND6127	WM1026,λsidA1,TR	53'	130'	+
ND6129	WM1026,λsidA1,TS	42'	65'	+
ND6138	WM1026,λsidA1,TS	42'	71'	+

prophage.

The results of Louarn *et al.* (pers. comm.), who have studied a phage which is apparently identical to λ*sidA*, provide an explanation for the two different types of λ*sidA* lysogen formed. They found that suppression by their phage was dependent on *recA*-dependent amplification of the prophage DNA. Clearly, this will occur only if an amplifiable duplication is available on which recombination can act to give unequal crossing over. Such will be the case in (a) dilysogens at *att*λ or (b) lysogens integrated by homology at *sidA*. Monolysogens at *att*λ will not be able to amplify, and will thus fail to suppress *dnaA*.

For this reason, it is doubtful whether the lack of an effect of λ*sidA1* on nalidixic acid resistance in CND18 reflects a genuine inability of λ*sidA1* to cause nalidixic acid sensitivity rather than a simple lack of significant overexpression of *sidA*, since, in a DnaA+ strain such as CND18, there is no selection for amplification, and therefore a monolysogen at *att*λ is the most probable type. It would be interesting to know what the effect of λ*sidA* on nalidixic acid resistance in a Nal^R,*dnaA* strain (where amplification can be selected) might be. It might be noted here that the *dnaA46* mutation itself reduces resistance to nalidixic acid (Filutowicz,1981).

recA-dependent amplification of λ*sidA1* offers an explanation of the wide range of growth rates recorded for the suppressed λ*sidA1* lysogen, ND61. It was sometimes noticed that after incubation of suppressed WM1026 lysogens at 30°C for some time, growth at 40°C produced cell lysis and that plating of cultures that had been maintained at 40°C resulted in a high frequency of small colonies which contained many elongated cells. This latter resembles the cold sensitive filamentation observed in ED419pND5 (which, apart from the different *sidA* episome, is isogenic to ND61). However, in the case of an amplifiable lysogen, adaptation to low or high temperature can easily be achieved as the result of unequal crossing over, or "looping out" of the duplicated segment of DNA, resulting in augmentation or diminution of *sidA* copy number as appropriate. Thus, in the case of ND61, it seems likely that

the variation in growth rate is a consequence of the different histories of the cultures used for the experiments. That this may result from quite subtle differences in history is shown by the fact that for two cultures of ND61 inoculated from single colonies on the same plate on successive nights and grown up overnight, the growth rates measured at 30°C on the following day were 100' and 58'.

For this reason also the apparent lack of any effect of λ*sidA* on cell division (see Fig. 3.6) needs to be reinvestigated, since the culture of ND61 used for this experiment was a rapidly growing one (doubling time = 58') while that used for the study on DNA replication (Fig 3.6) grew more slowly (doubling time = 77'). Preincubation of both these cultures at 30°C may have selected for diminution of λ*sidA* copy number, and therefore, the slow rate of DNA synthesis by ND61 on shift to 40°C might reflect the presence of a substantial non-suppressed subpopulation of cells in the culture. The shift from 40°C to 30°C might well give interesting results, both for DNA synthesis and cell division.

3.13 Mapping of the Chromosomal sidA Locus

In order to ask whether the SidA phenotypes can be correlated with the activity of any known genes, genetic mapping of the *sidA* locus was undertaken. Two methods for mapping cloned DNA fragments; the P1 transduction approach of Greener and Hill (1980) and the Hfr conduction method of Yamada and Hirota (1982) have given conflicting results. A new method of mapping has been developed in an attempt to resolve these difficulties.

Hfr-mediated conduction

The conduction method of Yamada and Hirota (1982) relies on the formation of transient cointegrates between the chromosome of an Hfr strain and high copy number plasmids carrying chromosomal homology. These homology-mediated cointegrates are transferred to the recipient during conjugation, and subsequently resolve. Thus, plasmid cloning

vectors which carry fragments of chromosomal DNA can be mobilised by Hfr strains and their antibiotic resistance genes serve as markers for the position of the otherwise cryptic chromosomal locus that they carry. Since conduction is an uncommon event relative to chromosome transfer, it is only detectable if the plasmid is transferred as an early marker.

Four Hfr strains: ED2433, ED3029, ED3062 and AB259, whose origins of transfer are shown in Fig. 3.8, were transformed with pND5 and pBR325 and the frequency of transfer of Tet^R to AB1157 was determined (Table 3.9).

All the Hfrs which transfer the region between 84' and 91' early transfer pND5, so a position within this region is indicated. However, these Hfrs have in common not only this region of early transfer, but also the presence of lac DNA close to their transfer origins (since they are all derived by integration of the lac F-prime factor $F42_{ts114}$). This DNA is reported to be transferred early by these Hfrs (C.Vermeulen, pers. comm.), although attempts to select lac+ exconjugants with these Hfrs gave very little transfer, possibly on account of the "early marker effect" (Glansdorff,1967). Thus the possibility that these Hfrs transfer pND5 by virtue of homology between lac and pND5 DNA must be considered. The only Hfr tested which fails to transfer pND5 is HfrH (AB259). This strain does not contain lac DNA as part of its F factor, but it should transfer lac as a moderately early marker (9' from the transfer origin). Thus it does not seem very likely that pND5 is transferred by homology to lac DNA.

P1 Transduction of pND5

There are two reports of the use of P1 transduction to map the chromosomal homology of ColE1-type cloning vectors with chromosomal inserts (Greener and Hill,1980; Silver and Wickner,1983). The procedure involves construction of a PolA strain transformed with the plasmid (a rare occurrence which can only occur by integration of the plasmid into the chromosome) and the preparation of a P1 transducing lysate on this strain, which can then be used in attempted cotransduction of plasmid

<u>Figure 3.8</u>

A : The plasmid pHR3

B : Genetic map of the region between the transfer origins of ED3029 and AB259, showing markers used in P1 transductions.

C: Genetic map of the *E.coli* chromosome, showing origins of transfer of Hfrs used in conduction experiments and relevant genetic markers.

Figure 3.8

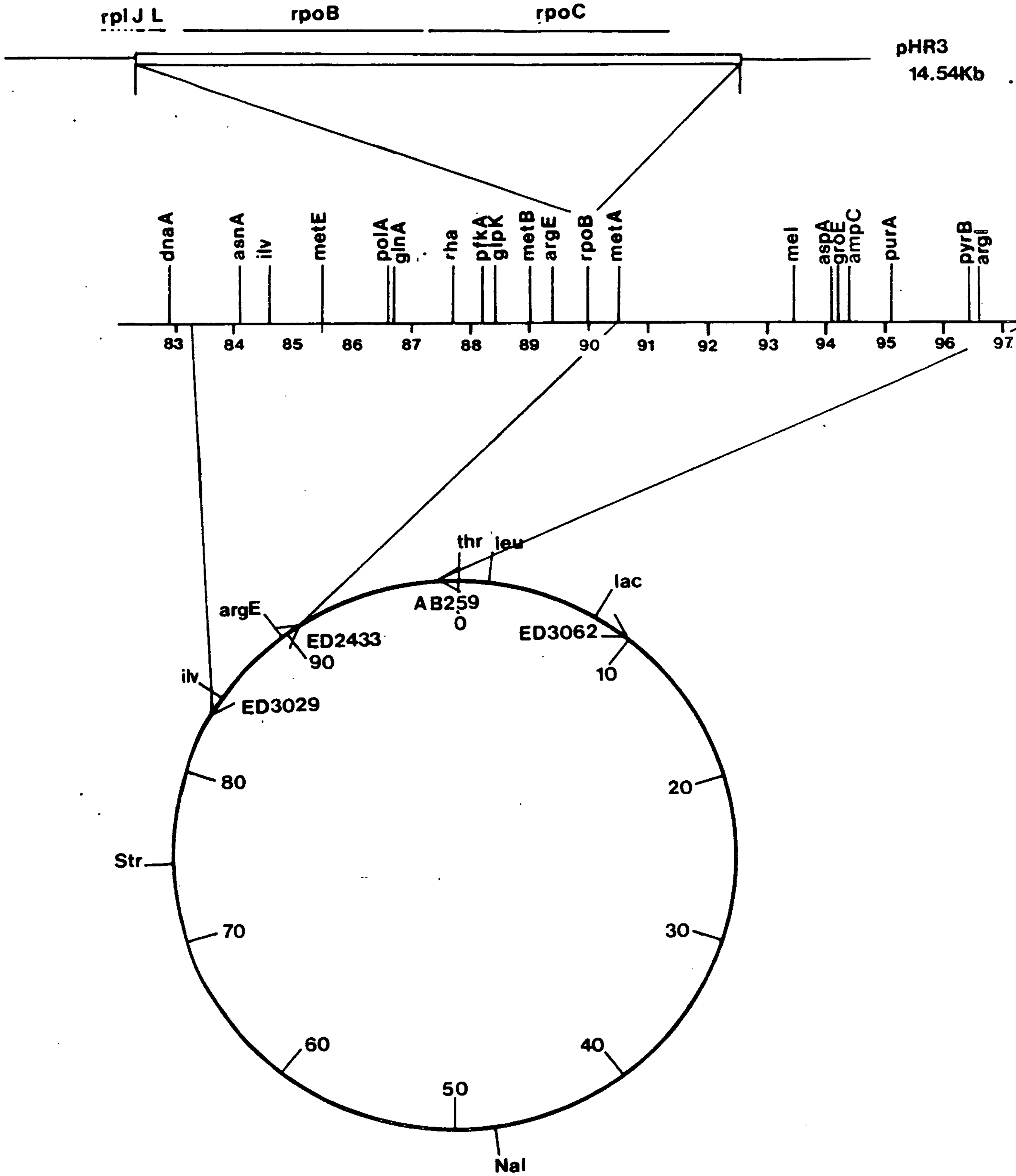


Table 3.9 Conduction frequencies of pND5 by various Hfr strains

Donor	Selected chromosomal marker/ distance from transfer origin	Transfer Frequencies after 30 mins tetR	chr	ratio x100	after 60 min tetR	chr	ratio x100
ND95(ED2433pND5)	*argH*/1′	218	5.02×10^{5}	0.044	160	1.46×10^{5}	0.011
ND98(ED2433pBR325)	*argH*/1′	4	5.2×10^{4}	0.008	-	-	-
ND96(ED3029pND5)	*argH*/7′	1190	1.16×10^{3}	0.73	860	3.08×10^{5}	0.28
ND121(ED3029pBR325)	*argH*/7′	22	1.2×10^{6}	0.002	20	7×10^{5}	0.003
ND97(ED3062pND5)	*thr*/10′	279	2.12×10^{4}	1.32	689	9.49×10^{4}	0.73
ND122(ED3062pBR325)	*thr*/10′	1	8.81×10^{3}	0.011	0	2.66×10^{6}	0
ND123(AB259pND5)	*thr*/1′	2	2.07×10^{5}	0.001	4	1.23×10^{5}	0.003
ND124(AB259pBR325)	*thr*/1′	2	1.84×10^{5}	0.001	0	7.97×10^{4}	0

"chr" indicates the selected chromosomal marker: *thr* or *argH*

antibiotic resistance with prototrophy using a set of auxotrophic recipients. If the site of homology of the plasmid is genetically linked to an auxotrophic marker, the plasmid and the marker will be cotransducible.

As this method is a new one, its efficacy was first verified by cotransducing a plasmid containing an insert of known chromosomal location. The PolA strain ND13 was transformed to Amp^R with the plasmid pHR3 which carries a 10kb insert of chromosomal DNA from λrifD18 including rpoB, rpoC, rplJ and rplL. This DNA comes from the 90' region of the E.coli chromosome. Fig. 3.8 shows this plasmid and its relation to the surrounding chromosomal genes.

A P1 lysate prepared on this strain (ND130) was then used to cotransduce pHR3 with the flanking markers metA and argH into the strain AB2569 (argH1,metA1,polA+). The results are shown in Table 3.10.

In addition to showing that pHR3 can be readily cotransduced with flanking markers, these results also show that it cannot be cotransduced with the unlinked marker proA. It is interesting to note that pHR3 is transduced more efficiently than the two flanking markers and that although it increases the physical distance between argH and metA by 10kb (appr 0.25') its presence actually increases their cotransduction frequency. In spite of this, when the Wu equation:

$$c = (1 - d/L)^3$$

where

c = cotransduction frequency

L = length of transducing DNA (taken by convention to be 2' (Bachmann,1983)

d = genetic distance between cotransduced markers in minutes

Table 3.10 Cotransduction of pHR3 with metA and argH

donor	selected phenotype	number of transductants	relative frequency	cotransduction frequency *					
				ar-me	me-ar	ap-me	me-ap	ap-ar	ar-ap
ND13	Met+	440	3.4						
	Arg+	1290	12.5						
	Met+,Arg+	72	0.69	23%	7.7%	-	-	-	-
	Pro+	103	1						
ND130	Met+	5860	22.0						
(ND13	Arg+	8390	31.4						
pHR3)	Amp^R	18870	70.7						
	Met+,Arg+	1710	6.4	29%	20%	-	-	-	-
	Met+,Amp^R	4050	15.2	-	-	69%	21%	-	-
	Met+,Arg+,Amp^R	1660	6.2						
	Arg+,Amp^R	5680	21.3	-	-	-	-	68%	30%
	Pro+	267	1						
	Pro+,Amp^R	0							

* Calculated from the frequency of the double transduction / the single transduction given as the SECOND marker. Thus me-ar indicate that the value was calculated from the number of Met+,Arg+ transductants / the number of Arg+ transductants

me = Met+ , ar = Arg+ , ap = Amp^R

was used to calculate genetic distances from these results, they were close to those expected (Table 3.11).

The presence of pHR3 also alters the frequencies of transduction of the two markers relative to each other and the unlinked proA marker. A rather similar effect is seen when the λrif transducing phage λ172, which carries the same insert as pHR3, is inserted into the chromosome. The results are compared in Table 3.12.

In the case of the RSH431 transduction, cotransduction is essentially abolished due either to zygotic induction of the prophage or its sheer physical size; but as with pHR3, the frequency of transduction of metA relative to argH is increased and apparently (although the control marker proA was not tested in the AJN30/RSH431 transduction) so also is the absolute transduction of markers in the proximity of the prophage. It is possible that this enhanced transduction results from replication of transducing fragments from the λ or pBR322 replication origin prior to recombination with the chromosome. Masters (1977) has suggested that the high frequency of transduction of markers close to oriC may have a similar explanation.

Alternatively, one might suggest that markers close to an integrated plasmid are preferentially packaged by P1, although this does not seem to be the cause of the high levels of transduction of markers close to oriC (M. Hanks, unpublished results). A possible contributory factor to the high levels of transduction of the plasmid itself is that since a plasmid might loop directly out of a transducing fragment a favourable recombination event resulting in integration of the selected marker into the chromosome is not necessary as it is with a non-replicating chromosomal marker. Thus, as many as 100% of recipients receiving the plasmid might become antibiotic-resistant.

It is interesting to note that when one compares the frequencies of transductants and cotransductants for donors with and without pHR3 (Table 3.10), most of the increase in transduction frequencies of metA and argH can be accounted for by the frequency of cotransductants

Table 3.11 Calculated* genetic distances based on data from table 3.10

	Donor	
	ND13	ND130
met - arg	0.77	0.63
arg - met	1.15	0.83
averaged	0.96	0.76
met - Amp	-	0.81
Amp - met	-	0.23
averaged	-	0.52
arg - Amp	-	0.66
Amp - arg	-	0.24
averaged	-	0.45

* From $D = 2(1 - \sqrt[3]{c})$ where c is the cotransduction frequency

The genetic distance between argH and metA is 1'. rpoB is equidistant between metA and argH.

Table 3.12 Effect of an Inserted Replicon on transduction of *metA* and *argH*

marker \ donor	AJN30(λ-)	RSH431(λ172)	ND13	ND130(pHR3)
metA	38	406	440	5860
argH	124	469	1290	8390
metA, *argH*	6	3	72	1710
proA	NT	NT	103	267

An overnight culture of AB2569 (*argH*,*metA*,PolA+,λ-) was concentrated 10x, 0.5ml was mixed with 1.5×10^{8} pfu of P1 transducing lysate and incubated at 37°C for 20'. The cells were harvested and resuspended in 2.5ml LB+2mM sodium citrate (NaCit) (ND13 and ND130 transductions) and incubated at 37°C for 45' before harvesting and resuspending in 2.5ml PBS+2mM NaCit and plating; or harvested and resuspended in this buffer directly (AJN30 and RSH431 transductions).

with Amp^R. Thus, for the transduction ND130 x AB2569, the frequency of Met+ transductants minus the frequency of Met+,Amp^R transductants is 22 - 15.2 = 7 (twice the frequency of Met+ transductants for the transduction ND13 x AB2569); and the corresponding frequencies for Arg+ and Arg+,Amp^R transductants give a difference of 31.4 - 21.3 = 10.1, slightly less than that observed in ND13 (12.5).

The very high ratio of argH to proA transduction, even from a strain lacking pHR3, is also notable. Newman (1982) found that proA was transduced at approximately twice the frequency of his, although she did not compare it directly with argH. his is transduced at a 4.4 fold lower frequency than argE, which is very closely linked to argH, so it would seem that the ratios for proA transduction reported here are significantly different from those usually found. That this is not a peculiarity of the recipient used is indicated by the fact that a similarly large ratio of purA : proA transductants is found using the recipient strain H1175 and an ND13 donor lysate. There are many differences between the donor strain ND13 and that used by Newman (W3110), but the polA mutation seems the most likely to be affecting transduction frequencies. Two possible explanations spring to mind for the very low relative transduction frequency of proA from ND13. One is that packaging specificity is altered in this strain. The other is that transducing DNA from a polA strain is in some way different in its susceptibility to the enzymes involved in recombination or DNA degradation, so that the proA DNA is either selectively degraded or the other markers tested are being selectively recombined.

Transduction of pND5

When 0.1μg of pND5 was transformed into ND13, 12 transformants were obtained, a frequency which is some 600-fold lower than that obtained in an isogenic PolA+ host. pHR3, which has a similar size of insert gave a similar frequency of transformation. One of these transformants, ND91, was used as a donor in P1 transduction experiments.

Table 3.13 summarises transduction data for Tet^R (pND5) and a range of markers in the 84'-91' region of the E.coli chromosome. The map locations of these markers are shown in Fig. 3.8.

Using the Wu formula, we can now calculate the minimum genetic distance between each marker and tet (ie the plasmid). The results are:

metA - tet	>1.5'
argH - tet	>1.75'
argE - tet	>1.5'
rha - tet	>1.2'
glnA - tet	>1.5'
ilv - tet	>1.4'
asnA - tet	>1.5'

Subtracting 0.3' to take account of the length of the plasmid, I infer that the pND5 homology cannot lie within the limits indicated by Table 3.14.

Thus, if we can assume that the Wu equation holds for experiments of this sort (and the data of Table 3.11 indicate that it does) the whole of the region between 83' and 91.7' seems to be eliminated as a possible sidA locus, although Hfr conduction suggests that sidA is between 84' and 91'.

A possible explanation for this apparent contradiction was afforded by the results of attempts to transduce the metE gene from ND91 into the metE strain χ478. Although a high frequency of Tet^R transductants was obtained, no transduction to Met+ was found in repeated experiments. Subsequent experiments showed that ND91 and its parent ND13 cannot be transduced to met+, although ND11, from which ND13 is derived can. All of these strains carry the metB1 mutation, but it appears that during the course of construction ND13 acquired a second met mutation. It seems likely that this arose as a deletion as a result

Table 3.13 Transduction of pND5 and Chromosomal Markers

Recipient strain	Selected Marker(X)	X+ Transductants	Tet^R Transductants	X+, Tet^R Transductants	Co-Transduction
AB2569	MetA+	298	1550	0	<0.016
	ArgH+	2180	2700	0	<0.002
AB1157	ArgE+	410	617	0	<0.012
MM304-2	Rha+	1000	83	0	<0.06
JG138(PolA-)	Rha+	310	56	0	<0.09
M5000	GlnA+	452	1300	0	<0.035
MM303	Ilv+	2820	225	0	<0.022
MM18	Asn+	487	888	0	<0.01

Maximum cotransduction frequency is calculated as 4.605/n, where n is the transduction frequency for the less well transduced marker of the pair. The number 4.605 is chosen as numerator in place of 0 since this is the mean of a population from which the binomial probability $p=(e^{-m}.m^{n})/n!$ of selecting a sample of size n=0 is 0.01.

Table 3.14 Limits on the Location of *sidA*

Marker	Position	Minimum Distance from *sidA*	Limits
asnA	84.1'	1.2'	82.9 - 85.3'
ilv	84.6'	1.1'	83.5 - 85.7'
glnA	86.7'	1.2'	85.5 - 87.9'
rha	87.7'	0.9'	86.6 - 88.6'
argE	89.5'	1.2'	88.3 - 90.7'
argH	89.5'	1.45'	88.1 - 90.0'
metA	90.5'	1.2'	89.3 - 91.7'

of curing the zif-1863::Tn10 insertion with which the polA mutation was introduced into ND11 since this insertion is reported to be close to metE (Kelley & Joyce,1983). ND91 is thus expected to carry a deletion of unknown extent in the metE region. Were this mutation closely linked to sidA, cotransduction of pND5 with chromosomal markers might be obscured due to transductants becoming simultaneously Met-. To test this, C600 (thr,leu,thi) was transduced to Tet^R using a P1 lysate on ND91, and Tet^R colonies were tested for methionine auxotrophy. Of 750 transductants tested, all were Met+. Evidently neither of the met markers of ND91 is closely linked to pND5.

Another possibility is that one of the transfer origins of the Hfr strains used in the conduction mapping had been misassigned. HfrH is a particularly well characterised Hfr with an origin of transfer between uxuA and valS (B.J.Bachmann, pers. comm.) and the origin of transfer of ED3029 has also been mapped precisely to the region between tna and bgl (83') (Masters,1975). Our knowledge of the origin of transfer of ED2433 is based on the observations that: (1) the strain, although derived from the Met+ strain ED2403, is Met-, and (2) that it transfers thr and leu late and argE and metB early (M.Masters, pers. comm.; and this work data not shown). On the basis of this information, the origin of transfer was assumed to be within the metA gene, the only gene between thr and argE whose inactivation could lead to a Met- phenotype. The possibility does, however, exist that the met mutation and the acquisition of the Hfr phenotype are coincidental. Furthermore, Louarn et al. (pers. comm.) had reported that a phage with properties very similar to λsidA carried the chromosomal groE genes, located at 94.2 minutes on the E.coli genetic map

Therefore, cotransduction of integrated pND5 with purA, melA and argI was attempted. The results of these transductions are shown in Table 3.15

Table 3.15 Cotransduction of pND5 with purA and melA

recipient	chromosomal marker (X)	X+ transductants	Amp^R transductants	X+Amp^R transductants
H1175	purA	118 (90)	326 (0)	34 (0)
H1175	argI/argF*	58 (0)	217 (0)	0 (0)
M2508	melA	231 (0)	1160 (0)	0 (0)

Figures given in brackets are for non-transduced control cultures

* argI and argF are duplicate genes, and therefore transductants receiving argI or the unlinked argF gene will be Arg+.

The absence of Mel+,Amp^R transductants is apparently an artefact of selection, for when a total of 4135 Amp^R transductants selected on MacConkey agar + melibiose + ampicillin were scored for the presence of red (Mel+) sectors, a total of 58 Mel+,Amp^R transductants were detected, giving a cotransduction frequency of 0.014; corresponding to a genetic distance of 1.52'. Details of the screening of melibiose transductants are given in Table 3.16.

Table 3.16: Cotransduction of mel and amp resistance

selected marker	number	comments	Mel+ MacConkey	Mel+ minimal	Amp^R MacConkey	Amp^R glucose	Amp^R melibiose
purA+	60	(H1175)				20	
Amp^R	4388	MacConkey	57				
	268	MacConkey		4			
	335	minimal	8				
	37	minimal		0			
Mel+	200	minimal			0		0

As Table 3.15 shows, it did not prove possible to select Mel+,Amp^R transductants directly on melibiose minimal medium + ampicillin. Neither were any Amp^R transductants found among 200 Mel+ transductants selected on melibiose-minimal. Selection of Amp^R on MacConkey or minimal medium gave 1160 Amp^R transductants per 0.1ml of transduced cells, of which 16 were Mel+. Selection of Mel+ gave 230 Mel+ transductants from the same quantity of cells. It follows that 16 out of the 230 Mel+ transductants should be amp^R. Selection for Mel+ therefore seems to select against recovery of Amp^R, although Mel+,Amp^R can be subcultured on melibiose-ampicillin minimal medium.

Assuming that the frequency of Mel+,Amp^R transductants has been correctly estimated on MacConkey mel-amp plates, and assuming that these are the only Mel+ transductants that have failed to grow on the minimal mel and mel-amp plates, we may estimate the cotransduction frequency of amp with mel to be $(1160 \times 0.014)/(231 + 1160 \times 0.014) = 0.066$, corresponding to a genetic distance of 1.19'. Table 3.17 shows the cotransduction frequencies and genetic distances calculated from the data of tables 3.15 and 3.16.

Table 3.17 Cotranscluction frequency of pND5 with mel and purA

	mel-Amp	Amp-mel	purA-Amp	Amp-purA
cotransduction	0.014	0.066	0.10	0.29
genetic distance	1.52	1.19	1.06	0.68
genetic distance (average)	1.36		0.87	

Subtracting from these distances the 0.3' that the 14 kb plasmid is expected to add to the genetic distance, the following genetic distances are obtained:

melA - sidA 1.06'
purA - sidA 0.57'

In view both of the inaccuracies that may have arisen due to the high reversion frequency of purA and and of the difficulties encountered in selection of Amp^R,Mel+ transductants, these figures must be regarded as very approximate. They do agree quite well, however, with the distance of 1.6' between purA and melA given in Bachmann,1983. This distance between the two markers precludes the possibility of sidA lying outside the interval between purA and mel. sidA appears to lie between purA and mel, at approximately 94.3', which is close to the position of the groE (mop) genes. The lack of cotransduction between argI and Amp^R is consistent with this conclusion.

It is interesting to note that unlike pHR3, pND5 does not appear to enhance the transduction of neighbouring markers, nor does it have a greatly enhanced transduction frequency relative to other chromosomal markers. (see Table 3.13 and Table 3.18). These results also show that

Table 3.18 : Transduction of markers neighbouring pND5 and pHR3

Marker	ND13		ND130(pHR3)		ND91	
	frequency	relative frequency	frequency	relative frequency	frequency	relative frequency
metA	440	3.4	5860	22	-	-
argH	1290	12.5	8390	31.4	-	-
proA	103	1	267	1	-	-
Amp^R	-	-	18870	70.7	122	24.4
purA	147	21	-	-	58	14.5
argI/argF	133	19	-	-	20	4
proA	7	1	-	-	5	1

Relative frequency is calculated relative to proA = 1

purA and argI are very highly transduced relative to proA and suggest that with polA donors, proA is a very poorly transduced marker.

The cotransduction frequencies for melA, purA and pND5 confirm the expected position of sidA between purA and mel. To investigate whether λsidA carries functional groE genes, this phage was tested for its ability to form plaques on a groE host. Wild type λ cannot grow on such hosts. The results of this test are shown in Table 3.19.

Table 3.19 : Growth of λsidA on a groE host

phage	host	
	CND20 (groE)	C600 (groE+)
λ 425 (dnaA)	-	+
λ 616	-	+
λsidA1	+	+
λsidA2	+	+

The test was done at 30° C and 42° C, and the results did not differ. λsidA1 and λsidA2 were also tested for their ability to complement the neighbouring aspA marker by a spot test, selecting for growth of a gltC,aspA- strain on 0.5% monosodium glutamate as sole carbon source. No growth was observed. Thus the 8.2kb sidA fragment carries groE and not aspA.

The gene to the right of groE is ampC, and the region around this gene has been physically mapped (Edlund & Normark,1979; Edlund et al.,1981). The size of the ampC-containing EcoR1 fragment is 5.7kb. Thus, the only known genes on the sidA fragment are mopA (groEL) and mopB(groES). Since it is not known which groE mutation is carried by CND20, it is possible that λsidA carries only one of these genes. Takeda and Hirota (1982) showed that dnaA suppression by their plasmid, pYT47, was dependent on the production of a 68kd protein. This is close to

the size (65kd) reported for the groE(L) protein (Hendrix and Tsui 1978).

Confirmation that sidA maps to the groE locus suggested that the origin of transfer of ED2433 might well have been misplaced since the proposed transfer origin (counterclockwise from 90.5') would give late transfer of groE (94.2'), whereas this Hfr is able to mobilise pND5 as if this plasmid carried an early marker. To check this, cross-streak matings were performed between ED2433 and NF279rec+ (metB,ilv,pyrB), MM311 (mel, ilv), and H1175 (purA,argI). These results showed that ED2433 transfers metB and ilv early, but not pyrB, argI, purA, or mel, so its transfer origin lies between 89' (metB) and 93.4'(mel), and the assumption that the F-prime is inserted in the metA gene is probably correct. It is difficult, therefore, to account for the ability of ED2433 to mobilise pND5. Limited homology between sidA and the lac region is one possibility; the superficial similarity between mel and lac which encode α-galactosidase and β-galactosidase respectively, and also encode interchangeable galactoside permeases (Prestidge and Pardee, 1965), lends some credibility to this suggestion. It would be interesting to explore the ability of lac F-primes to mobilise pND5.

3.14 R46-tra mediated Conjugation

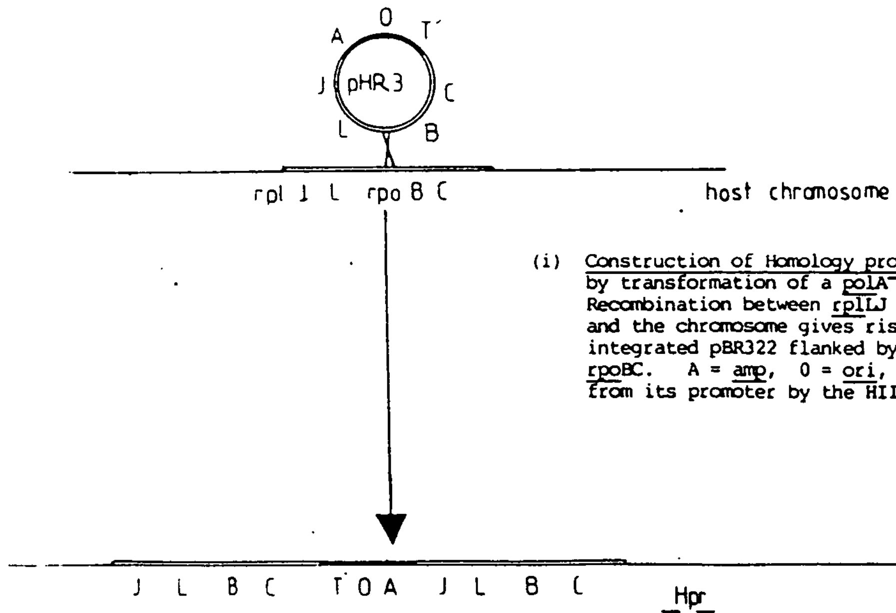
In the course of attempts to resolve the problems of mapping the sidA locus, a new method of mapping was developed, based on the availability of pBR325 derivatives carrying the transfer origin of R46 (Brown & Willetts,1981). The essentials of this technique, which should be applicable to any cloned E.coli DNA sequence in a pBR325 or related vector, are shown in Fig. 3.9.

In addition to allowing precise location of the chromosomal locus of a cloned sequence by defining this sequence as the point of origin of an Hfr (Hpd) strain, since the direction of transfer from R46 oriT is known (Coupland and Willetts, manuscript in prep.) it is possible also to determine the orientation of the fragment in the E.coli chromosome. This has been used to orientate the sidA fragment (see below).

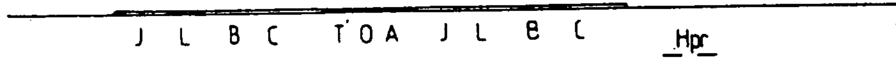
Figure 3.9

In this figure, recombination between the chromosome, pHR3 and pED935 leading to an "Hpd" strain, with Hfr-like properties is described.

Figure 3.9



(i) Construction of Homology probe recipient [Hpr] strain by transformation of a polA⁻ host with pHR3. Recombination between rplLJ - rpoBC DNA on the plasmid and the chromosome gives rise to chromosomally integrated pBR322 flanked by a duplication of rplLJ - rpoBC. A = amp, 0 = ori, T' = tet gene separated from its promoter by the HIII insert.



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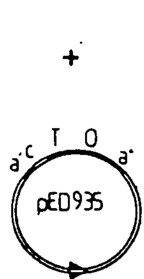
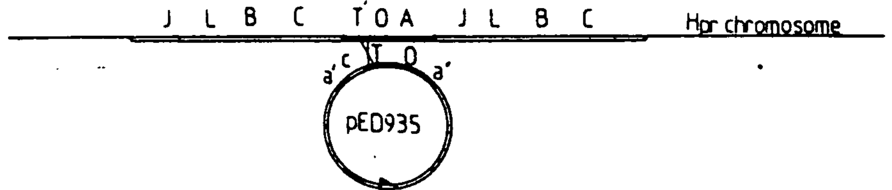
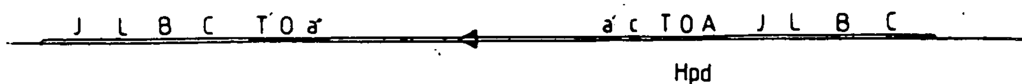


(ii) Construction of Hpd strain by mating the self-transmissible pED935 into Hpr. Recombination between pBR322 DNA of pHR3 and pED935 results in the integration of the R46 transfer origin into the chromosome at the centre of a complex duplication. a', a" = amp gene of pBR322 interrupted by pstI R46 tra insert, C = Cm^R insert of pBR325, T = complete tet gene of pBR322.



(iii) Hpd strain; the chromosome is transferr from ⟶ at 90' on the E.coli map. The strain will transfer rplJL, rpoBC [90'] then metA (90.5'), metE [94'] thr 100/0' etc.



Transfer of the R46_tra_-pBR325 recombinant plasmids pED935 and pED936 (which both carry the transfer origin and all the transfer genes of R46, but in opposite orientations) into the _polA_,pND5 strain ND91 must result in the formation of an Hfr strain with its origin of transfer at _sidA_, since pED935 and pED936 cannot replicate in a _polA_ host and have no chromosomal homology except for that provided by the pBR325 moiety of pND5, with which they must recombine in order to be maintained. Fig. 3.9 illustrates the basis of this construction.

The construction was carried out as follows: ND91(_polA_,pND5), ND13(_polA_) and ND11(_polA_+) were mated with ED535 (pED935) and ED536 (pED936) on membrane filters at 37°C for 30 mins in a ratio of 10 recipients to 1 donor and Cm^R exconjugants were selected. (This selection is made possible since pED935 and pED936 have their inserts in the _amp_ gene of pBR325 while pND5 has _sidA_ inserted into its _cat_ (chloramphenicol resistance) gene. The results are shown in Table 3.20

Table 3.20 Transfer of pBR325/R46tra recombinants

	Cm^R exconjugants per 10^7 viable recipients		
	ND11 (PolA+)	ND13 (PolA-)	ND91 (PolA-,pND5)
donor			
ED535	4.8×10^6	1×10^2	9×10^3
ED536	6.4×10^6	0.44×10^2	6.8×10^3

Comparing the values for ND11 and ND13 it appears that between 0.12% and 0.14% of ND91 exconjugants form a chromosomally integrated cointegrate between pND5 and pED935 or pED936. Since the available homology for integration is 6kb, this frequency compares well with that for transformation of pND5 (0.17% via 8.2kb of homology) into PolA+ and

PolA- backgrounds.

ND91,pED935 (ND132) and ND91,pED936 (ND133) can be used in cross-streak matings in the same way as conventional Hfr strains. Table 3.21 shows the results of such an experiment.

No transfer of chromosomal markers was observed from isogenic polA+ strains carrying pED935.

Since these markers all lie to one side of the melA - purA region (Fig. 3.8), these results are consistent with the results of P1 transduction.

These results allow the orientation of the sidA locus in the chromosome. The reasoning used here is most easily explained diagrammatically by drawing the three elements of the Hpd strain superimposed as part of a screwlike structure: the mobilising plasmid (pED935), the adaptor plasmid (pND5), and the host chromosome (Fig. 3.10A). Once this structure is drawn, with the arrow indicating oriT of R46 drawn in so as to indicate transfer of the appropriate chromosomal markers, the known orientation of oriT within pED935 is used to locate reference points on pED935 and pND5: in this case, the tet and amp genes (Fig. 3.10B). Finally, the known position of an asymmetrically placed restriction target within the pND5 insert is drawn in on both chromosome and plasmid (Fig. 3.10C).

In this diagram, the plasmids have been distorted to bring the tet and amp genes into line with one another while retaining the transfer origin in the most illustrative position at the top of the figure. The "correct" diagram gives the same result, but is rather less easy to follow (Fig 3.10D).

These Figures also show that the simplest way of representing the structure is to include the transfer origin and insert on a single plasmid molecule. Combining mobilising plasmid and adaptor in the same molecule, besides being a useful diagrammatic simplification, is a genuine practical

Table 3.21 Transfer of Chromosomal Markers By sidA/Hpd Strains

Donor	selected marker				
	ilv	*glnA*	*pfk*	*glpK*	*argE*
ND132 (pED935)	+	+	+	+	+
ND133 (pED936)	-	-	-	-	-

Figure 3.10

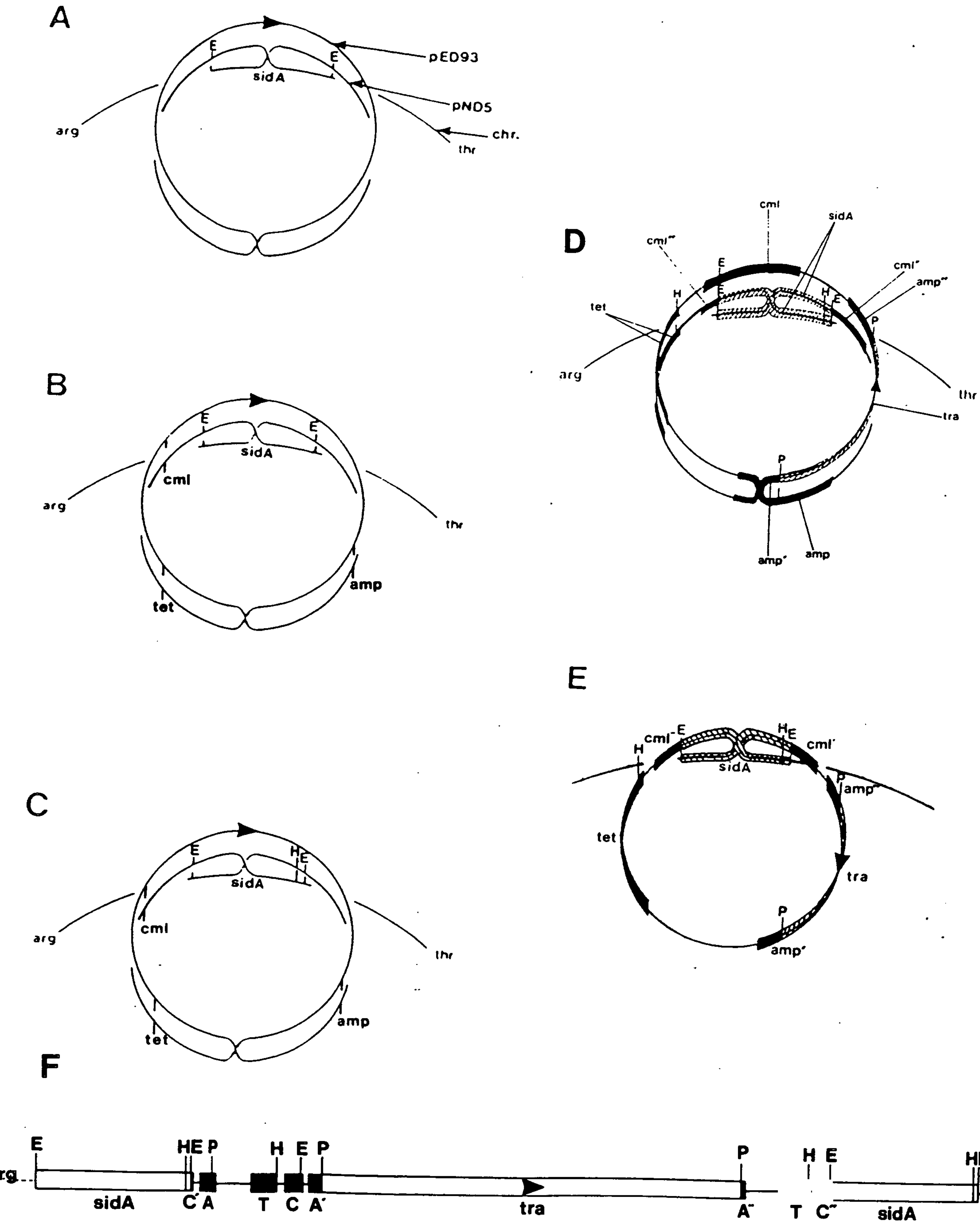


Figure 3.10 : Determination of the chromosomal orientation of sidA

A : The plasmids and chromosome are drawn, and the transfer origin positioned to indicate the correct orientation relative to chromosomal markers transferred.

B : The diagram is "orientated" by drawing in the positions of plasmid markers by reference to the transfer origin..

C : The position of an asymmetrical site (the HindIII site in sidA) is ascertained by reference to the plasmid markers.

D : Figure C redrawn with all markers in their correct positions. Insert DNA is indicated by hatched boxes, vector antibiotic resistance genes are indicated by filled boxes. Interrupted genes are indicated thus: "cml'", "amp''" etc.

E : Simplified version of figure D with one copy of pBR325 removed by a hypothetical recombination event. Use of symbols as in D.

F : D drawn as a linear structure. Filled boxes are plasmid antibiotic resistance genes, open boxes are insert DNA. T = tet, A = amp, C = cml

E = EcoR1
P = PstI
H = HindIII

possibility. The transfer region of R46 has very few restriction targets and thus pED935 and pED936 have unique EcoR1, BamH1 and HindIII targets like their parent vector pBR325, so subcloning a fragment of interest into this vector should prove relatively straightforward. Alternatively, an *in vivo* recombinant between pED935 and a plasmid carrying the fragment of interest could be quite readily obtained by standard procedures.

Construction of a "mobiliser-adaptor" plasmid would obviate the need to transform the adaptor plasmid into a *polA* strain (which appears to be the most troublesome step in the procedure), since the mobiliser-adaptor is transfer-proficient and can thus be introduced by mating.

Such a plasmid could be used in the same manner as F-prime plasmids for chromosome transfer, and, being more easily selected (due to the available antibiotic-resistance markers), might in many circumstances be preferable to F-primes. It may not be necessary to perform chromosome mobilisation from a *polA* background, since Coupland and Willetts (manuscript in prep.) have shown that high copy number plasmids carrying R46 transfer functions and Tn5 can mobilise the *E.coli* chromosome when this also contains a Tn5 insertion, and that this will occur in a *polA*+ background.

Thus, this approach has two potential uses:

1) Precise chromosomal localisation of a cloned fragment.

2) Chromosome mobilisation from a chosen position when, for some reason, Hfr or F-prime mediated mating is not desirable.

3.15: Comparison of sidA with neighbouring sequences

The availability of a chromosomal orientation for the *sidA* fragment prompts a comparison between its restriction map and that of the neighbouring *ampC* region.

The leftward boundary of the chromosomal insert in pNU1, a plasmid isolated from the Clark-Carbon library (Edlund et al,.1979), which carries an insert of 12kb of DNA from the ampC-frd region, appears to overlap sidA, since it contains a HindIII-EcoR1 fragment of 290bp, the same size, and with the same orientation of restriction sites as the right hand HindIII-EcoR1 fragment in sidA. pNU1 carries a further 1.5kb of DNA to the left of the HindIII-EcoR1 fragment, and this contains no sites for HindIII, EcoR1, BglII PstI or XhoI, which is also the case for the corresponding region of sidA (Fig 3.3 and Takeda & Hirota,1982). Subject to confirmation by direct comparison of pNU1 and sidA, I propose the restriction map shown in Fig. 3.11 for the 18.4kb frdA-ampC-groE region.

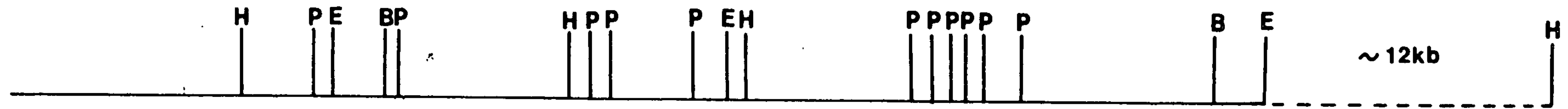
Fig. 3.11 indicates that a 2.7kb HindIII fragment of E.coli chromosomal DNA should hybridise to the 8.2kb sidA EcoR1 fragment. In Fig. 3.2 only 1 fragment that does not correspond to a known λ-homologous band (Kaiser & Murray,1979) can be detected in the hybridisation between HindIII-digested C600 DNA and λsidA1. Its size is approximately 20kb and it is presumably the HindIII fragment overlapping the 7.9kb left hand HindIII-EcoR1 fragment. Considering the rather low level of hybridisation obtained in this experiment, it is not surprising that no fragment overlapping the 290bp HindIII-EcoR1 fragment could be detected.

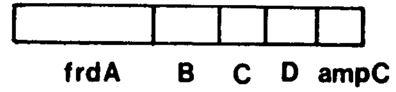
3.16 Concluding remarks

The results presented in this chapter demonstrate suppression of dnaA by groE or a gene closely linked to it. The formal possibility does exist that sidA is a suppressor not only of dnaA, but also of groE; but the closeness of their map positions, and the similarity in size between the groE and sdaA (pYT47) products, makes this a most unlikely possibility.

The observation that high copy number suppressors can be active when cloned in λ, due to recA-dependent amplification of the recombinant prophage, demonstrates the importance of using recA hosts when screening phage libraries for recombinants complementing a gene of

Figure 3.11





groE

1kb

Figure 3.11. Restriction map of the frdA-ampC-groE region of the E.coli Chromosome

P = PstI

E = EcoR1

H = HindIII

B = BglII

The dotted line indicates that the disposition of EcoR1, PstI and BglII sites in that region is not known.

The map is based on Edlund _et al_.,(1979), Takeda & Hirota (1982), and this work.

interest. Two other groups (Louarn *et al.*, pers comm.; Rowen *et al.*,1982) have also found *dnaA* suppressor genes cloned in λ, and it seems likely that other examples will emerge for other genes. It would be interesting to know whether the *dnaA* suppressor phage isolated by Rowen *et al.* is also a λ*groE*, since this phage was isolated from a *Salmonella typhimurium* library.

However, had the sensible precaution of using a *recA* strain been taken this fascinating and unexpected interaction between *groE* and *dnaA* would have been overlooked. I shall return to the possible mechanisms of *dnaA* suppression by *groE* in the discussion (Chapter 6).

Chapter 4. Conservation of Sequences in the Region of the Origin of Replication

4.1. Estimation of Sequence divergence

Two major pathways of genome evolution are recognised: vertical evolution, due to the accumulation of mutations under the influence of selection and genetic drift; and lateral evolution, due to exchange of mobile genetic elements. Lateral evolution has attracted considerable interest of late, since the best example of it, the spread of antibiotic resistance in bacterial populations is of considerable medical importance. The existence of lateral evolution presents difficulties in microbial systematics since the possibility of the phylogeny of bacteria being a net rather than a tree undermines the validity of the relationship between the hierarchical system of classification, and the supposed phylogeny of the species classified.

In some cases, such as antibiotic resistance genes carried by plasmids and transposons, the evidence for lateral evolution is abundantly clear (Anderson,1968); while in the case of housekeeping genes such as those required for protein synthesis, ribosome manufacture, and central metabolic processes, there is no special reason to expect lateral transfer to occur. How much of evolutionary change in bacteria is due to lateral transfer and how much to vertical evolution remains a matter for conjecture.

An approach to resolving this problem would be to adapt the DNA/DNA hybridisation method (Brenner,1973) which has found much favour in bacterial taxonomy as a means of comparing whole genomes, to a gene-by-gene analysis. For a given gene, the actual amount of sequence divergence between two species will vary depending on the characteristic rate of sequence evolution of that gene (slow for conserved sequences, rapid for non-conserved sequences). If evolution of the sequence is vertical, a constant factor in the similarity of that sequence in various

species will be the evolutionary relatedness of those species (which is assumed to be directly related to the time since divergence), the sequence difference being:

$$D = 2mt$$

Where m is the characteristic mutation rate, t is the time since divergence of the two species and D is the sequence divergence in basepairs. If a bacterium A is more closely related (more recently diverged) to species B than to C, all genes of A will be more similar in sequence to those of B than those of C, in constant proportion, according to the formula

$$D_c/D_b = t_c/t_b$$

In other words, if all species are compared pairwise for each gene, and the species then ordered in a matrix of similarity, the position of each species in that matrix should be the same for whatever gene is under consideration.

This relationship will hold only if the sequences diverge at a constant rate in the different species, and if there is no lateral transfer of genes. (Note that in the equations given above, the factors which correct for the reduced rate of divergence due to back-mutations and mutations in already diverged bases have been omitted for the sake of simplicity.)

It follows from this that, although there is no obvious way of distinguishing lateral transfer from differential rates of sequence evolution, it should be possible (by using gene-by-gene) comparisons, to ascertain the proportion of genes whose evolution is vertical, and constant in rate, and thus to gain an estimate of the maximum contribution of lateral evolution in bacteria.

The origin of chromosome replication is interesting as a potential laterally transferred sequence. Its DNA sequence is known to be highly conserved in a number of enteric bacteria and in the marine Vibrio species, V.harveyi (Zyskind et al.,1983). On the other hand, a number of findings suggest that oriC has the potential for involvement in lateral transfer.

The function of oriC can be replaced by that of other low copy number replicons (integrative suppression, Nishimura et al.,1971) or, in rnh mutants, by otherwise cryptic origins (Kogoma & von Meyenburg,1983; de Massey et al.,1984), which indicates that under suitable circumstances oriC could be lost and/or replaced by an unrelated (or distantly related) origin. The sequence of oriC is related to that of the replication origins of bacteriophages G4 and λ (Meijer et al.,1979) and the plasmid pSC101 (Yamaguchi & Yamaguchi,1984), which suggests a common evolutionary origin and a potential for interspecific transfer of oriC-related replicons. OriC can be maintained as a plasmid, albeit with rather low stability (Messer et al.,1978; Yasuda & Hirota,1977). Taken together, these findings suggest that as an alternative to simple vertical inheritance the chromosomal origins of gram negative bacteria may follow a path of evolution which involves alternation between function as a chromosomal origin and function as a phage or plasmid origin, with interspecific dissemination in the latter state.

To explore this possibility, I have used hybridisation to Southern transfers (Southern,1975) of genomic DNA from various members of the Enterobacteriaceae and related gram-negative bacteria with probes derived from oriC itself, sequences contiguous to oriC, and unlinked DNA sequences.

In order to analyse these data quantitatively, I have developed a method for assessing the intensity of bands on autoradiograms in the form of a " percentage hybridisation" result.

It was originally hoped that it would prove possible to apply the restriction fragment matching method of Upholt (Upholt,1977). This method relies on the fact that related sequences will have similar restriction patterns and that the frequency of similar fragments can be used to estimate sequence similarity. However, for this method to be accurate, it is essential that only point mutations should occur; a deletion could remove several restriction targets in a single step. Furthermore, the frequency of matched restriction fragments could also be altered if, due to small size, some fragments are not retained on the filter; or if some fragments, due to rapid sequence divergence, are not detected by hybridisation.

The major difficulty in applying Upholt's method to gene-by-gene comparisons is, however, that Southern transfers can reliably detect fragments only down to approximately 500bp. Below this size, retention on nitrocellulose filters declines (Southern,1975). In a gene-by-gene comparison, it is clearly desirable to use a probe consisting of a single gene or operon, but since such sequences are likely to be in the 1kb-10kb range, it is not possible to generate a sufficient number of fragments of suitable size for the frequency of identical fragments to be estimated with any degree of confidence.

I therefore sought to obtain quantitative data from the intensity of hybridisation observed. This approach affords less complete data than restriction fragment matching could do, since it is only possible to assess similarity to the probe sequence, rather than to all other species for which fragments are detected.

Other workers have applied hybridisation to Southern transfers to the question of sequence conservation of various bacterial genes (Anilionis & Riley,1980; Ostupchuk _et al_.,1980; Bohnert _et al_.,1980; Wright and Boyle,1984), but these authors have been content to assess the conservation of sequences by visual examination of their autoradiograms, or they have attempted to use Upholt's method to estimate sequence divergence.

When the probe is in excess, the amount of radioactive probe DNA bound in a filter hybridisation reaction will be proportional to the abundance of the filter-bound complementary sequence and to the stability of the duplex formed and thus to the sequence similarity between the probe and the filter-bound DNA. In bacteria, where repetitive DNA is not common, the abundance of complementary DNA sequence is a function of genome size and the amount of genomic DNA bound to the filter. Thus, if we can determine the amount of filter bound DNA and the intensity of the hybridisation signal, we obtain a measure of sequence similarity between probe and the sequence to which it hybridises. This is essentially the technique employed when taxonomic affinities between bacterial species are compared by whole-genome DNA/DNA hybridisation (Brenner,1973) or rRNA/DNA hybridisation (De Smedt et al.,1980); except that in these cases, the cold filter-bound DNA is in excess over the probe.

4.2. Estimation of DNA concentration

In a Southern hybridisation, filter-bound radioactivity can be rather easily assessed by scanning an autoradiogram with a densitometer and integrating the peaks obtained. Reliable estimation of DNA concentration is more difficult. DNA concentration is normally estimated by measuring the absorbance of a solution at 260nm. However, genomic DNA of the high molecular weight suitable for restriction digestion and Southern transfer is often very viscous, and as a result, reproducibly accurate dispensing of the small volumes necessary is not practicable with an automatic pipette.

For this reason, I preferred to measure the DNA concentration in the gel in situ, by applying the same densitometric method used for determining the intensity of bands on an autoradiogram to a photograph of the ethidium bromide stained gel, and converting these measurements to micrograms of DNA by comparison with a DNA standard of known concentration (HindIII-digested λcI857).

A simpler approach was also tried, where agarose containing 5µg/ml of ethidium bromide was set in a petri dish, restriction-digested DNA samples (5µl) were spotted on and the fluorescence compared by eye with that of a dilution series of the same HindIII-digested λ DNA used for standardisation in the densitometric procedure. Results obtained by all three methods with the DNA used in fig.4.4B are compared in table 4.1. Comparison with the gel suggests that the results obtained by measuring A_{260} are inaccurate, probably on account of difficulties in accurate dispensing of the highly viscous stock solutions, while the most accurate results appear to be those obtained in the spot tests. Scanning of the gel photograph seemed to produce results of adequate accuracy, but appears to be less sensitive; so that high values appear to be overestimated and low ones underestimated. This is probably in part due to difficulty in establishing a suitable baseline for integration of scans of negatives that have a significant background. Nonetheless, since values obtained by the spot test were not available for all the restriction digests used in these experiments, I preferred the results of scanning gel photographs when processing the data since this allowed a consistent method to be used. DNA concentration determined by scanning the gel photograph was generally in better agreement with visual estimates from the same photograph than the data of table 4.1 suggest.

Table 4.1 suggests that in addition to its quickness, simplicity and very small consumption of sample, the spot test method is remarkably accurate; and is therefore, in many cases, preferable to spectrophotometry, especially when it enables the DNA concentration in working samples, rather than in stock solutions, to be estimated.

4.3. Quantitation of hybridisation signal

When analysing autoradiograms it was in many cases not possible to compare the intensities of bands directly since either (a) the standard band (E.coli) was so much more intense than the band in question that the settings on the densitometer had to be adjusted to obtain

Table 4.1 Comparison of three methods of DNA estimation, using gelB of figure 4.4.

DNA Concentration (μg/ml)

DNA	Track	Scan	Spot	A_{260}
H.alvei	2	4.0	4	1.9
E.aerogenes	3	3.4	6	1.4
E.cloacae	4	2.8	3	1.9
K.pneumoniae	5	2.4	1.6	5.5
S.marcescens	6	2.2	1.6	5.6
P.alcalifaciens	7	3.4	4	2.0
P.stuartii	8	2.3	3	2.6
M.morgani	9	1.8	3	2.7
P.vulgaris	10	3.2	6	1.3
V.natriegens	11	1.5	1.6	5.4

Spot tests: 5μl samples of EcoR1-digested DNA in restriction buffer were spotted onto 1% agarose gel in Tris-acetate gel buffer containing 10μg/ml ethidium bromide in a plastic petri dish and examined on an ultraviolet transilluminator.

Gel Scan: photographs of the ethidium bromide stained gel (see Fig 4.4) were scanned with a Joyce-Loebel microdensitometer and the area under the trace compared to a standard 1μg of HindIII digested λ DNA.

A_{260}: stock DNA solutions were diluted in TE buffer and absorbance was measured at 260nm and 280nm in a 400μl 1cm pathlength quartz cuvette. 260:280 ratios were greater than 1.8.

measurable deflections for both; (b) the band in question was so much more faint than the standard band that two different exposures of the autoradiogram had to be used; or (c) the band in question was on a different filter from the standard band.

In all these cases, I employed an "intermediate standard": a band whose intensity was measurable on both exposures of the autoradiograms or at both densitometer settings. The relative intensity (m/s) of a measured band of intensity m_1 measurable only on exposure 2 of an autoradiogram was calculated by reference to the intensity (i_1,i_2) of an intermediated standard, measurable on both exposure 1 and exposure 2, and the intensity (s_1) of the reference standard (*E.coli*), measurable only on exposure 1. Thus:

$$\text{relative intensity} = m/s = m_2/i_2 \times i_1/s_1$$

An essentially identical procedure was employed when the band to be compared was not on the same filter as the standard. In this case, it was possible to take advantage of the presence of contaminating λ-homologous DNA in the probes, which hybridised to the λ standards. Since the same quantity of λ DNA was applied to the gels, and the same probe was used for both filters, the λ standards served as useful intermediate standards between the filters.

4.4. Bacterial species studied

The species studied are listed in table 4.2. Most of the species tested belong to the *Enterobacteriaceae*. *Vibrio natriegens* belongs to the *Vibrionaceae* (the next most closely related family (Baumann and Baumann,1977)). The rest belong in the broad category of the Pseudomonads, a large group whose taxonomy is in a state of flux. I have tried to group together the species which have the closest relationships, drawing on the work of De Smedt *et al*.(1980), Auling *et al*.(1980), De Ley *et al*.(1978) and De Smedt & De Ley (1977). The

groupings must, however, remain conjectural and considered as a guide rather than as a well founded taxonomic grouping. All the bacteria studied were gram-negative.

Salmonella typhimurium is not included in this list since it was not possible to analyse the results obtained with this species. The DNA prepared from S.typhimurium was apparently highly sheared or degraded (see Fig. 4.3), although it was still possible to observe a ladder pattern when EcoR1-digested DNA was electrophoresed, and thus hybridisation to this DNA resulted in a smear (probe 9.8 and probe A) or no detectable hybridisation (OriC). Consequently, it was not possible to estimate the size of homologous fragments or the strength of the hybridisation signal.

In Fig. 4.5, DNA from Shigella sonnei was used. This species is closely related to E.coli (see Fig. 4.1). Since only the one probe was used on this species, it is not possible to use the data comparatively, and the results are not considered here.

In table 4.2, the species of the Enterobacteriaceae have been grouped together according to the dendrogram of Brenner,1984, which is shown in fig. 4.1. The biochemical characteristics of these strains are given in table 4.3.

4.5. Results

Figure 4.2 shows the extent of the probes used and the genetic loci encoded, where known. For brevity, these are called "OriC" (the 400bp oriC-containing fragment), "pCM959" (an in vivo constructed minichromosome composed of 4kb of DNA surrounding oriC and including the asnA gene (Meijer et al.,1979), "probe 9.8" (the 9.8kb oriC-containing EcoR1 fragment, which also carries asnA, gidA, gidB, and part of the atp (unc) operon), "probe A" (an unidentified chromosomal insert cloned in λ616) and "pPM2000", a pBR325 derivative carrying a 10kb insert of DNA from the region of the E.coli replication terminus. Probe A was originally thought to be the 8.2kb EcoR1 insert from λsidA, but the

Table 4.2: Species studied

Group1: Enterobacteriaceae

Escherichia coli

Citrobacter freundii

Enterobacter aerogenes
Enterobacter cloacae
Klebsiella pneumoniae

Morganella morgani

Providencia alcalifaciens
Providencia stuartii

Serratia marcescens

Proteus vulgaris

Hafnia alvei

Group2: Vibrionaceae

Vibrio natriegens

Group3 : other gram negative bacteria

Pseudomonas aeruginosa
Pseudomonas putida

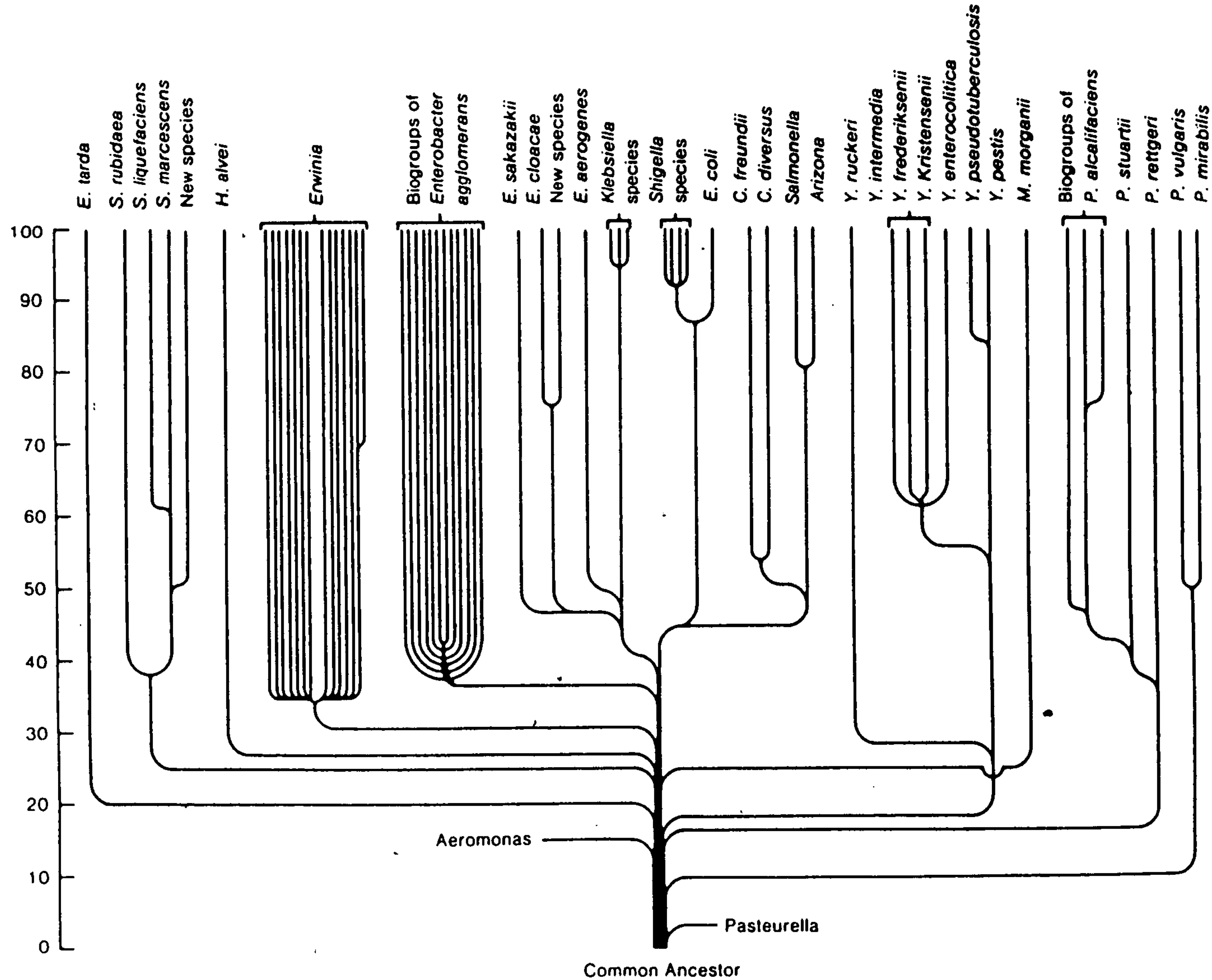
Chromobacterium violaceum
Alcaligenes faecalis

Rhizobium leguminosarum
Azotobacter vinelandii

Figure 4.1. Dendrogram of the Enterobacteriaceae.

Based on whole genome DNA - DNA hybridisation. From Brenner(1984).

Figure 4.1



Divergence of *Enterobacteriaceae*. The *ordinate* is percentage of relatedness. This figure is a simplified attempt to depict relatedness of each species of enterobacteria to all other species. It assumes a common ancestor from which all of the organisms have diverged. The horizontal branches depict the degree of relatedness of the group of organisms to all organisms that have not yet branched. For example, *E. tarda* is ~20% related to all organisms except *Aeromonas*, *Proteus*, *Providencia* and *Pasteurella*; *Citrobacter* species are ~45% related to all species above them and *C. diversus* and *C. freundii* were speciated at a point in time such that they are now 50% related.

Table 4.3. Results of biochemical tests

SPECIES	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU
Alcaligenes faecalis	-	-	-	-	-	-	-	-	-	-	-	-
Citrobacter freundii	+	+	-	-	+	+	-	-	-	-	-	+
E.coli ND11	+	-	-	-	-	-	-	-	+	+/-	-	+GF
Enterobacter aerogenes	+	-	+/-	+	+	-	-	-	-	+	-	+
Enterobacter cloacae	+	+	+/-	+	+	-	-	-	-	+	-	+
Hafnia alvei	+	-	+	+	-	-	-	-	-	+/-	-	+
Klebsiella pnuemoniae	+	-	+	-	+	-	+/-	-	-	+	-	+
Morganella morgani	-	-	-	+	+	-	+	+	+	-	-	+/-GF
Proteus vulgaris	-	-	-	-	-	+	+	+	+	+	+	+G
Providencia alcalifaciens	-	-	-	-	-	-	-	+	+/-	-	-	+F
Providencia stuartii	-	-	-	-	-	-	-	+	+	+/-	-	+
Pseudomonas aeruginosa	-	+	-	-	+	-	+/-	-	-	-	+	-
Serratia marcescens	+	-	+	+	+	-	-	-	+/-	+	+	+

ONPG: B-galactosidase ADH: arginine dihydrolase LDC: Lysine decarboxylase ODC: Ornithine decarboxylase H2S: Production of hydrogen sulphide URE: Urease TDA: Tryptophan deaminase IND: Tryptophanase VP: Voges-Proskauer GEL: Gelatin liquefaction GLU: Utilisation of glucose

Table 4.3. Results of biochemical tests

MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	CAT	NO2	MOB	MAC	OF-O	OF-F	SCORE
-	-	-	-	-	-	-	-	+	+	+	+	.	.	.	43%
+G	+(F)	+	+	+	-	+F	+	-	+/-	+	+	+	+	+	0.083%
+G	-	+F	+/-F	-	-	-	-	-	-	+	+	+	+	+	0.03%
+(F)	+	+	+	+	+	+	+	-	+/-	+	+	+	+	+	70%
+G	-	+F	+	+	+	+F	+	-	+/-	+	+	+	+	+	54%
+F	-	-	+F	-	-	-	-	-	+	+	+/-	+	+	+	1.7%
+(F)	+	+	+	+G	+	+G	+	-	+/-	+	-	+	+	+	45%
-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	63%
-	-	-	-	+	-	+	-	+/-	+	+	+	+	+	+	6%
-	-	-	-	+F	-	-	-	.	+	+	+	.	.	.	0.05%(1)
+F	+F	-	-	-	-	-	-	.	+	-	-	.	.	.	0.00034%(2)
-	-	-	-	-	-	-	-	+/-	+	+	+	+	+	-	3%
+	+F	+F	-	+	-	+	-	-	+	+	-	+	+	+	6.4%

Sugar utilisation tests: MAN = mannose; INO = inositol; SOR = sorbitol; RHA = rhamnose; SAC = sucrose; MEL = Mellibiose; AMY = starch; ARA = arabinose. OX: oxidase CAT: catalase NO2: Reduction of nitrate to nitrite MOB: motility MAC: Growth in MacKonkey medium OF-O: oxidative metabolism OF-F: Fermentative metabolism SCORE: percentage of members of this species expected to have this biochemical profile. Calculated from the manufacturer's table of percentage positive results for each test.

(1) Score for Proteus vulgaris = 0.023%

(2) Score for Proteus retgeri = 0.0003%

F: Sugar utilisation primarily fermentative. G: Gas produced

Figure 4.2

S H X E E

asnA 17kd 16kd ori gidA gidB I atpB F E atpH atpA atpG atpD C

-3 -2 -1 0 1 2 3 4 5 6 7 8 9

oriC

pCM959

probe 9.8

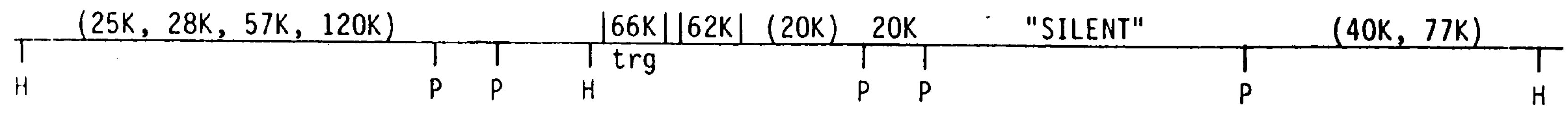


Figure 4.2 : Chromosomal regions surrounding the probes used.

Top : The oriC region: genes and relevant restriction targets. The figure is calibrated in kb. Position 0 is the same as position 0 in fig 1.4 and is the BamHI target considered by convention to be position 0 of oriC. The orientation is inverted relative to the conventional orientation of the E.coli genetic map. From von Meyenburg et al.(1980), Gay & Walker (1981) Saraste et al.(1981), Buhk & Messer (1983).

Bottom : The trg region (between minutes 31 and 32 on the E.coli genetic map. Sizes of polypeptides encoded by this region are indicated. (From Moir and Masters, manuscript in prep.). The positions in kb are from the physical map of the E.coli replication terminus region (Bouche,198?).

E = EcoR1 , H = HindIII , P = PstI , S = SmaI , X = XhoI

pattern of restriction fragments lit up in an EcoR1 digest of C600 chromosomal DNA (Fig. 4.5) did not include an 8.2kb fragment, and was not similar to that obtained in the hybridisation shown in Fig. 3.2. Evidently, a different fragment of DNA had been used in the preparation of probe A. The results obtained with this probe were nonetheless included in the analysis since they provide a useful comparison to those obtained with oriC-containing probes.

Table 4.4 shows results (relative intensity and fragment sizes) for the 3 oriC probes: the oriC region probe, probe 9.8 (9.8kb), the minimal origin probe, OriC (400bp), and the minichromosome pCM959 (4012bp); table 4.5 shows the results for probe A. The autoradiograms for OriC and probe 9.8 are shown in fig. 4.3 and fig. 4.4 and those for probe A are shown in fig. 4.5. Since neither an E.coli control nor size standards were available for the pPM2000 hybridisation, fragment sizes and relative intensities could not be esimated for the bands homologous to this probe. Hybridisation intensity corrected for DNA concentration and arbitrarily standardised is given in table 4.6.

No species outside the Enterobacteriaceae show detectable homology to the oriC region. Assuming that the V.natriegens oriC is as homologous to E.coli oriC as that of V.harveyi (70%, based on the 290bp sequence reported by Zyskind et al.,1983) it seems that this hybridisation system only detects fragments with >70% homology to the probe.

4.6. Localisation of conserved regions around oriC

In E.coli, a single EcoR1 fragment of 9.8kb lights up with all oriC probes. Three additional bands of 11.8kb, 16.6kb and 20.3kb can be explained as the products of partial digestion. Von Meyenburg et al. (1980) have reported that the EcoR1 fragments in the oriC region are (reading from left to right in the orientation of Fig. 4.2) 13.3kb, 9.7kb, 2.6kb and 4.8kb. This would account for the additional bands as follows:

11.8kb: 2.6 + 9.7 = 12.3

Table 4.4 Fragments Homologous to OriC region Probes

Species	probe 9.8 size/kb	probe 9.8 relative intensity	pCM959 size/kb	pCM959 relative intensity	oriC size/kb	oriC relative intensity
Escherichia coli	20.3	0.086				
	16.6	0.045				
	11.8	0.019				
	9.6	1	9.5+/-0.2	1	9.8	1
Citrobacter freundiii			13.8	*		
			10.4	*		
	9.0	0.2	8.3	5.1	9.8	0.085
	3.25	0.76				
Hafnia alvei	10.4	0.042	9.5+/-0.14	0.15	0	0
Enterobacter aerogenes	8.7	0.51	-	-	7.2	0.25
					2.1	0.0019
					1.9	0.0012
Enterobacter cloacae	15.1	0.44	-	-	19.8	0.094
					10.7	?
	5.6	0.043				
Klebsiella pneumoniae	15.1	0.21	15.8+/-3.1	0.27	19.8	0.043
					10.7	0.029
Serratia marcescens			4.9	0.17	4.2	0.0051
	2.64	0.19				
	1.75	0.026	1.6	0.06		
Providencia alcalifaciens			-	-	5.4	0.0051
					5.2	0.0051
					4.7	0.0019
	2.64	0.05				
Providencia stuartii	2.64	0.12	-	-	-	-
Morganella morganii			-	-	7.1	*
					4.8	*
					10.7	*
	2.64	0.10				
Proteus vulgaris	9.8	0.064	9.6+/-0.05	0.42		
					3.1	*
					3.2	*

* Intensity of band too low for measurement. - Not tested .0 No detectable homology

Figure 4.3 : Hybridisation of probe 9.8 to Bacterial genomic DNA

A1 : Genomic digests of:

1 λcI857 (HindIII)
2 *E.coli*
3 *Salmonella typhimurium*
4 *Citrobacter freundii*
5 *Alcaligenes faecalis*
6 *Pseudomonas aeruginosa*
7 *Psedomonas putida*
8 *Chromobacterium violaceum*
9 *Rhizobium leguminosarum*
10 *Azotobacter vinelandii*
11 λcI857 (HindIII)

B1 : Genomic digests of:

1 λcI857 (HindIII)
2 *Hafnia alvei*
3 *Enterobacter aerogenes*
4 *Enterobacter cloacae*
5 *Klebsiella pneumoniae*
6 *Serratia marcescens*
7 *Providencia alcalifaciens*
8 *Providencia stuartii*
9 *Morganella morgani*
10 *Proteus vulgaris*
11 *Vibrio natriegens*
12 *Chromobacterium violaceum*
13 λcI857 (HindIII)

Except where otherwise stated, all digests are with EcoR1.

A2, B2 : The same gels transferred to nitrocellulose and hybridised with probe 9.8 (A2, tracks 2-10; B2, tracks 2-12) or λcI857 (A2, tracks 1 + 11; B2, tracks 1 + 13).

The sizes of lambda standard bands are indicated

Faint bands are marked by arrows

Fig. 4.3

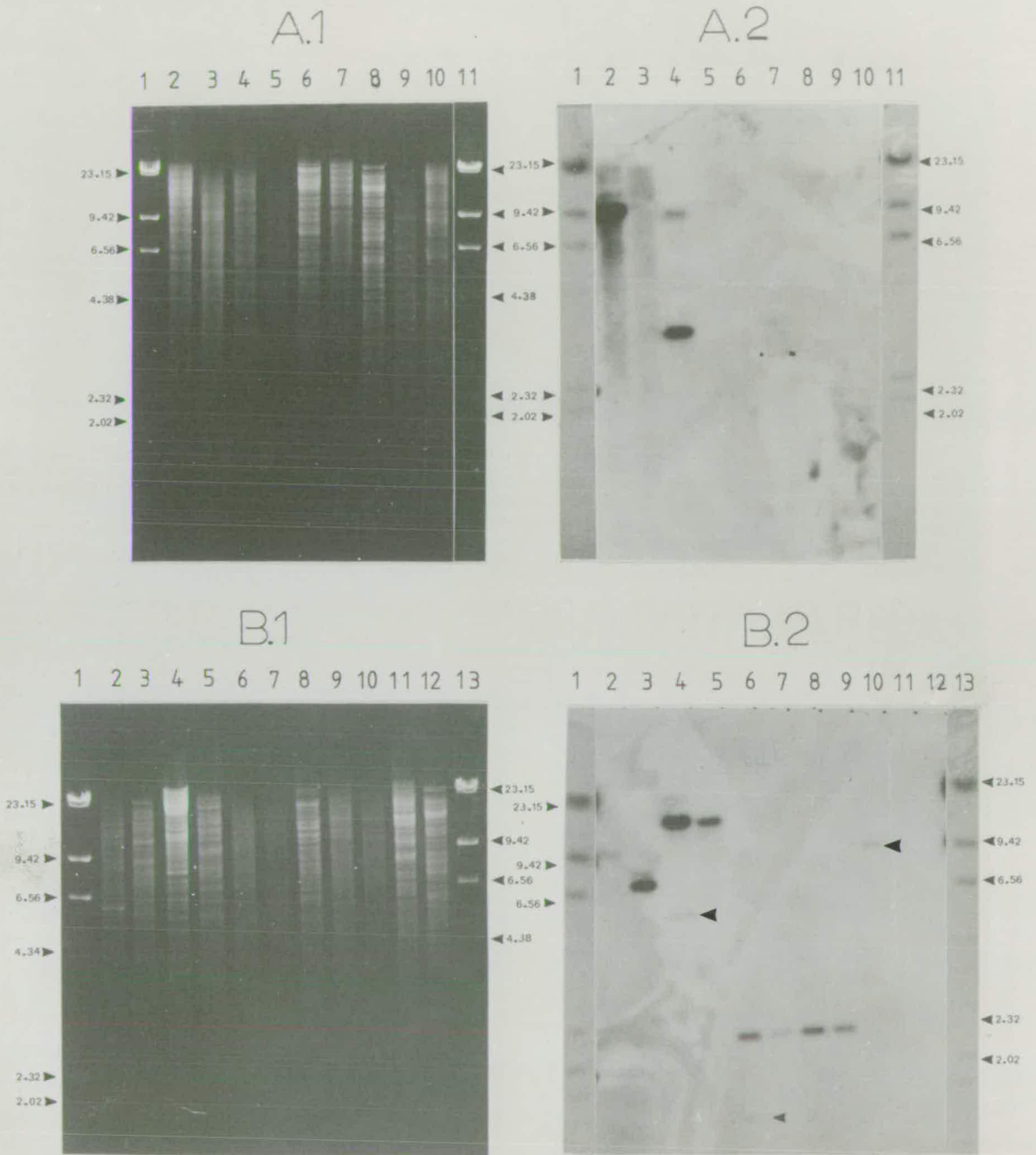


Figure 4.4 Hybridisation of bacterial genomic DNA to oriC

A1 : Genomic digests of:

1 λcI857 (HindIII)
2 *Bacillus subtilis*
3 *Azotobacter vinelandii*
4 *Rhizobium leguminosarum*
5 *Chromobacterium violaceum*
6 *Pseudomonas putida*
7 *Pseudomonas aeruginosa*
8 *Alcaligenes faecalis*
9 *Citrobacter freundii*
10 *Salmonella typhimurium*
11 *E.coli*
12 λcI857 (HindIII)
13 pCM959 (XhoI + SmaI)

B1 : Genomic digests of

1 λcI857 (HindIII)
2 *Hafnia alvei*
3 *Enterobacter aerogenes*
4 *Enterobacter cloacae*
5 *Klebsiella pneumoniae*
6 *Serratia marcescens*
7 *Providencia alcalifaciens*
8 *Providencia stuartii*
9 *Morganella morgani*
10 *Proteus vulgaris*
11 *Vibrio natriegens*
13 λcI857 (HindIII)

Except where otherwise stated, all digests are with EcoR1

A2, B2 : The same gels transferred to nitrocellulose and hybridised to oriC probe (A2, tracks 2-11; B2, tracks 2-12) or λcI857 (A2, tracks 1 + 12; B2, tracks 1 + 13)

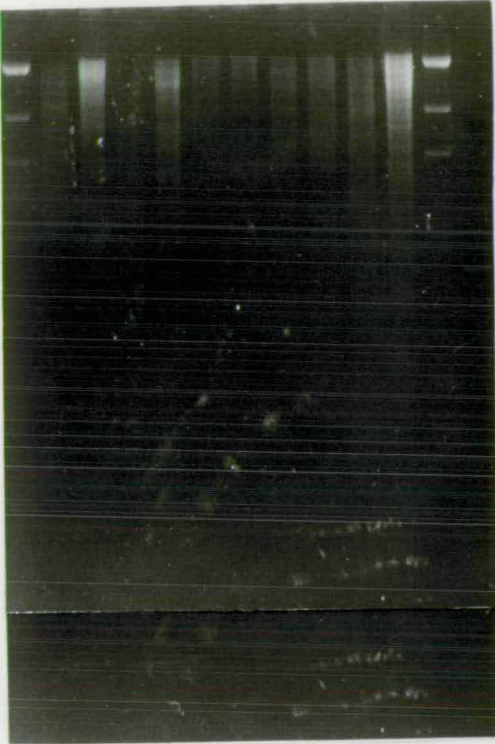
Faint bands, not visible in this reproduction are marked by arrowheads.

The sizes and positions of λ size standards are indicated.

FIG 4.4

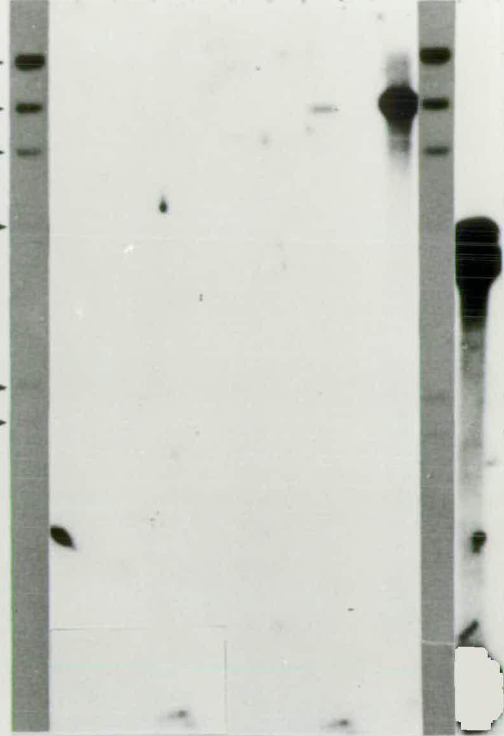
A1

1 2 3 4 5 6 7 8 9 10 11 12 13



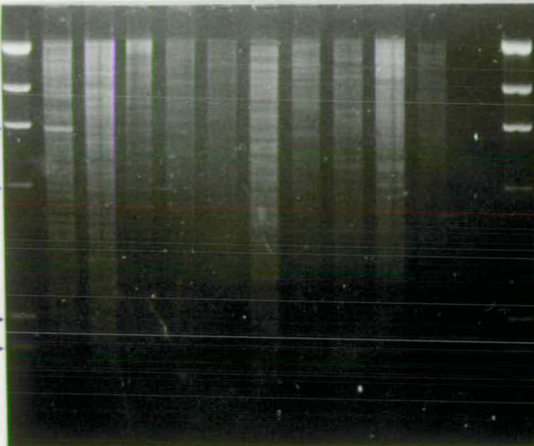
A.2

1 2 3 4 5 6 7 8 9 10 11 12 13



B.1

1 2 3 4 5 6 7 8 9 10 11 12 13



B.2

1 2 3 4 5 6 7 8 9 10 11 12 13

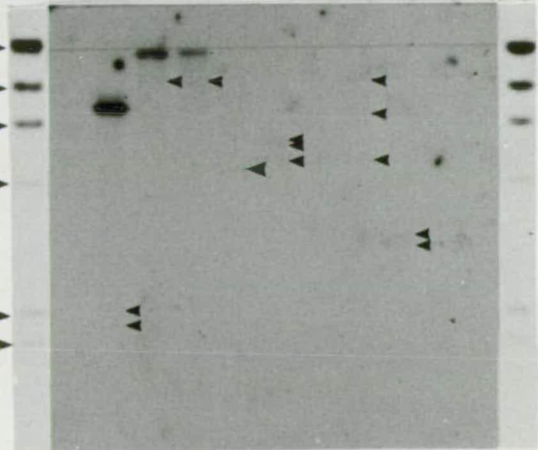


Table 4.5 EcoR1 Fragments Homologous to Probe A

Species	size(kb)	intensity
E.coli	15.7	0.91
	12.3	*
	10.9	0.0087
	9.3	0.036
	6.2	0.022
	3.0	0.027
C.freundii	4.4	0.03
	3.6	0.025
	2.7	0.009
E.aerogenes	5.9	0.02
	3.8	0.1
	2.5	0.019
	1.9	0.067
E.cloacae	5.9	0.15
	6.3	0.007
	4.3	0.003
	2.5	*
S.marcescens	14.2	0.002
	4.4	0.004
P.alcalifaciens		0
P.stuartii	7.4	0.0007
M.morgani		0
P.vulgaris	7.4	0.022
H.alvei	20.2	0.021
	11.9	0.016

0 : No homology detected
* : Intensity not measured; band either too faint or formed a shoulder of a more intense band.

Figure 4.5. Hybridisation of genomic DNA to probe A

	Track	Species
A1	1	*Pseudomonas aerogenes*
	2	*Alcaligenes faecalis*
	3	*Citrobacter freundii*
	4	*E.coli*
	5	λcI857
B1	1	λcI857 (HindIII)
	2	*Shigella sonnei*
	3	*Azotobacter vinelandii*
	4	*Rhizobium leguminosarum*
	5	λcI857 (HindIII)
C1	1	λcI857 (HindIII)
	2	*Hafnia alvei*
	3	*Enterobacter aerogenes*
	4	*Enterobacter cloacae*
	5	-
	6	*Serratia marcescens*
	7	*Providencia alcalifaciens*
	8	*Providencia stuartii*
	9	*Morganella morgani*
	10	*Proteus vulgaris*
	11	*Vibrio natriegens*
	12	λcI857 (HindIII)

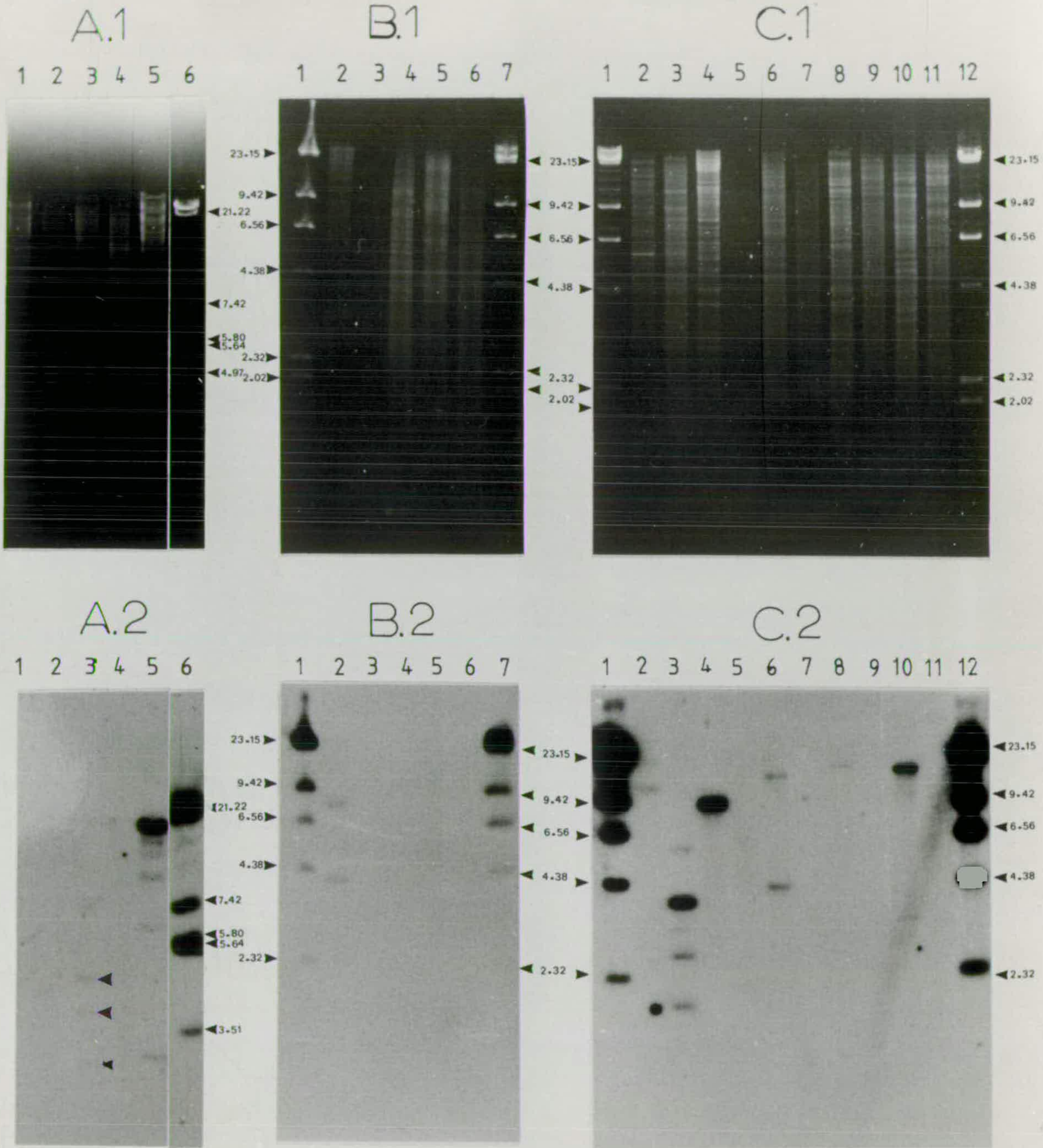
Except where otherwise stated all digests were with EcoR1.

A2, B2, C2: These gels transferred to nitrocellulose and probed with probe A.

The positions of faint bands are indicated by arrows.

DNA of *Shigella sonnei* was a kind gift of George Coupland. The sizes of the bands hybridising to this DNA are 7.8kb and 4.0kb. Since data for this probe alone are available with *S.sonnei*, results for this species are not discussed in the text.

FIG 4.5



16.6kb: 4.8 + 2.6 + 9.7 = 17.1

20.4kb: 9.7 + 11.8 = 21.0

The 9.8kb fragment is the chromosomal asnA-oriC-gid-atp fragment located at 84' on the genetic map of E.coli (Bachmann,1983).

In Citrobacter freundii, all three probes light up a band slightly smaller than the E.coli oriC fragment, of approximately 9kb (estimates varied between 9.8 and 8.3kb), whose homology to OriC seems rather feeble, but which gives a strong signal with pCM959.Probe 9.8 lights up an additional fragment of 3.25kb which, in spite of its smaller size, gives a fourfold stronger signal than the oriC-containing fragment. Thus, it appears that in this as in other species (see below) oriC is one of the less conserved sequences in the oriC region; and that another region, present on probe 9.8 but not on pCM959 or OriC, is the most highly conserved DNA. The two homologous regions may be contiguous in C.freundii, since pCM959, which does not light up the 3.25 kb fragment, lights up a fragment measured as 13.8 kb in a partial digest of C.freundii DNA, whose size could be explained as the sum of a 9kb and a 3.25kb fragment.

In Klebsiella pneumoniae, a large fragment of 15-19kb (the gel system and size standards used here are not suitable for measuring such large fragments with any degree of accuracy) hybridises to all three probes, but most weakly to OriC. Klebsiella pneumoniae has well-founded taxonomic affinities with the genus Enterobacter (Brenner,1984), and the pattern observed in the less complete data for Enterobacter cloacae supports this; although Brenner (ibid.) has pointed out that Ent. aerogenes, which gives a different restriction pattern, is more closely allied to Klebsiella than is Ent. cloacae. In both K.pneumoniae and Ent. cloacae, a very weakly hybridising fragment of 10.7kb is detected by OriC, one of a number of such fragments detected by this probe alone in various members of the Enterobacteriaceae. That this fragment is detected only by OriC is probably a reflection of the very long exposures used in this experiment; the 10.7kb band having an intensity

of only 1/300 of that of the E.coli oriC band. The only difference in the hybridisation pattern of Ent. cloacae and K.pneumoniae is the presence of a 5.6kb band of 1/10 the intensity of the 15kb fragment which hybridises to probe 9.8 in Ent. cloacae but not K.pneumoniae, though the different amounts of DNA loaded onto the gel could mean that this fragment is simply too faint to detect due to the lower concentration of DNA in the K. pneumoniae track (Fig. 4.3).

In these two species, it seems likely that most of the homology to the chromosomal region between atp and asn in E.coli is confined to a single fragment, and that the sequences are thus contiguous.

These data, so far as comparison can be made, agree with those derived from physical and genetic analysis of cloned origins of replication from Ent. aerogenes and K.pneumoniae (Harding et al.,1982). The size of the EcoR1 fragment carrying oriC in Ent. aerogenes SD1 is 7.1kb, a value consistent with the results presented here for strain NCTC 13048 of between 7.2 and 8.7kb. The atpB and asnA genes of Ent. aerogenes SD1 are carried by a SalI fragment of 18kb to which the 7.1kb fragment is entirely internal. The restriction map of Harding et al suggests that the organisation of DNA in this region is similar to that in E.coli but that the rightward EcoR1 site should separate asnA from the fragment containing oriC and the atp genes, and so the 7.1kb fragment should not carry asnA. Since, with the exception of two very faint bands homologous to OriC, only this single 7.2-8.3kb fragment is lit up by OriC (which lacks asnA), and by pCM959 and probe 9.8 (which carry asnA), it could tentatively be suggested that the conserved DNA in Ent.aerogenes lies to the left of asnA. However, it is possible that asn homology in Ent. aerogenes resides in EcoR1 fragments so small that they migrate off the end of the gel and are thus not detected, an explanation that could always be advanced to account for the lack of homology to a particular region of DNA instead of that of lack of evolutionary conservation.

If the most conserved DNA in this set of species is the same sequence, it seems likely, for the following reasons, that this DNA is

from the atp operon:

1) The failure of probe 9.8, which carries asnA, to light up any band in Ent. aerogenes DNA other than the 7.2kb band, which, if it is equivalent to the 7.1kb fragment studied by Harding et al. (1982), lacks the asnA sequence and the DNA beyond this gene, suggests that the region of this probe which carries the asnA gene and the DNA beyond does not carry any highly conserved DNA.

2) The most conserved DNA (the strongest bands) in C.freundii (3.25kb) and Serratia marcescens (2.64kb, see below) are homologous to probe 9.8, but not to pCM959 or OriC. They are therefore homologous to the DNA which is included on probe 9.8 and not on pCM959 (see Fig. 4.2). This DNA includes the DNA beyond asnA, which the reasoning of (1) above suggests is not highly conserved, and the gid - atp region; by process of elimination, it would seem, therefore, that this latter segment of DNA contains the most conserved sequences in the 9.8kb region of DNA studied here.

It is interesting to note in this context that Krebbers et al. (1982) have reported that the β subunit of the F_1 sector of the membrane coupling factor of maize chloroplasts is highly homologous to the product of the atpD gene of E.coli, its functional homologue. The EcoR1 fragment used here does not carry the atpD gene and carries only one of the genes for the F1 sector of the atp complex, atpH, which codes for the δ subunit. It is not known whether the F_0 sector, all of whose subunits are encoded by the 9.8kb EcoR1 fragment is similarly highly conserved.

Unfortunately, the 10.2kb K.pneumoniae SalI oriC fragment which Harding et al. describe contains only one EcoR1 site, so fragment size comparison with the 15 - 19kb EcoR1 fragment found in K.pneumoniae here is not possible, except insofar as the data are not inconsistent. The EcoR1 site in their fragment appears to cut the atpA gene (as it does in E.coli), and the atpB,F,E & H genes, oriC, and asnA are all

contained within the EcoR1-SalI fragment, as my data would predict. This does, however, call into question the significance of the 5.6kb Ent. cloacae fragment homologous to probe 9.8; for if, as it seems, the restriction patterns of DNA homologous to the oriC region is the same as in K.pneumoniae, and all the oriC region functions are encoded by the 19kb EcoR1 fragment, the 5.6kb fragment (unless it is an artefact of the digestion) would appear to represent a duplication of some of this DNA.

Since the EcoRI fragment containing the origin of replication of Salmonella typhimurium is 19.4kb (Zyskind & Smith,1980), and this is approximately the same size as the fragment observed for K.pneumoniae and Ent. cloacae (but not C.freundii, which is more closely related to S.typhimurium), it would be interesting to compare these species.

In Serratia marcescens, probe 9.8 lights up a 2.64 kb fragment that is common to this species, Prov. alcalifaciens, Prov. stuartii and M.morgani. In addition, in Serratia alone, a second band of 1.7kb is seen and this fragment hybridises to both pCM959 and probe 9.8, but not to OriC. OriC lights up neither of the above fragments, but instead hybridises weakly to a fragment of 4.4kb. This allows some conclusions to be drawn about the regions around oriC to which the various fragments are homologous. The 2.64kb band is homologous to DNA lying outside the boundary of pCM959 in the ori region probe and is probably a fragment of the atp operon, since the DNA between the right hand end of asnA and the right hand EcoR1 site in probe 9.8 contains no known genes. The 1.7kb fragment is within pCM959 and not OriC and so is homologous to DNA to the right (16kd,17kd genes,asnA) or to the left of oriC (gid). Apparently this DNA is not conserved in M.morgani, or either of the Providencia species. Together, the 4.4kb, 2.64kb and 1.6kb fragments add up to 8.7kb, so the possibility that they are all contiguous and arranged in Serratia as they are in E.coli is quite conceivable.

4.7. Evidence for multiple copies of oriC

In *Prot.vulgaris*, a band of a size indistinguishable from that in *E.coli* hybridises to both pCM959 and probe 9.8. This fragment is not, however, lit up by OriC. Instead, 2 very faint bands of 3.1 and 3.2kb are detected. That these bands are not detected by the larger probe is probably a consequence of the shorter exposure times employed when this latter probe was used. Thus, although a fragment containing DNA from the origin region is apparently conserved in size, it no longer carries detectable homology to *oriC*. The presence of bands apparently homologous to OriC but not to probe 9.8 and *vice versa* is a feature of many of the species more distantly related to *E.coli*. However, it should be stressed that the apparent lack of homology of *oriC*-homologous fragments to probe 9.8 is probably an artefact due to very faint bands being revealed by the long exposure times employed for the OriC filter, since all the sequence on OriC is contained in probe 9.8.

In *Providencia alcalifaciens*, three bands, of 4.8, 5.2, and 5.4kb hybridise to OriC, while only one, of 2.64kb, hybridises to probe 9.8; in *M.morgani*, the same 2.64kb fragment hybridises to probe 9.8 while fragments of 7.1, 4.8, and 10.7kb hybridise to OriC. Multiple bands homologous to OriC are also discernible in *Ent. aerogenes*, where two very faint bands of 2.1 and 1.9kb are lit up by this probe and in *K.pneumoniae* and *Ent. cloacae*, where OriC lights up an extra 10.7kb fragment although the data of Harding *et al*.(1982) suggest that all the *oriC* region genes are contained on the larger, more strongly hybridising 15kb fragment.

In *H.alvei* and *P.stuartii*, no homology to *oriC* was detected. In *H.alvei*, pCM959 and probe 9.8 light up a fragment of about 10kb.

It is interesting that so many species should seem to have multiple copies of sequences homologous to *oriC*, since if these are active, it would suggest that these (unlike *E.coli*) initiate chromosome replication at multiple sites. However, the fact that the two faint *oriC*-homologous

fragments detected in Ent. aerogenes NCTC13048 and the extra 10.7kb band in K.pneumoniae and Ent. cloacae were not detected when Harding et al. (1982) cloned the replication origins of Ent. aerogenes SD1 and K.pneumoniae, suggests that these sequences might not be active as origins although the possibility that these extra bands are absent from the strains used by Harding et al. due to strain differences must be born in mind. One may ask, therefore, what is the function (if any) of these duplicate oriC sequences in Ent. aerogenes? One possibility is that like the origins of replication used in constitutive stable DNA replication (De Massey et al.,1984), these are duplicate origins used for certain types of inducible replication, and hence are unable to replicate in normal E.coli strains. It is not as yet known whether the origins used in stable DNA replication have any homology to oriC. Examination of Fig. 4.4A shows that the presence of additional oriC-homologous bands in E.coli DNA is a possibility; two bands of can be seen in the 9.4kb to 6.6kb region of the track, but since these are seen against a high level of background, presumably caused by chromosomal contamination of the probe DNA, it is possible that these bands are simply a reflection of the ladder pattern of EcoR1 digested E.coli genomic DNA. Another possibility is that the multiple oriC-homologous sequences are remnants of now disused replication origins, or the remains of oriC-related replicons.

It would clearly be of interest to attempt to clone these weakly homologous oriC sequences and investigate their ability to replicate in E.coli, although perhaps a more urgent consideration is to attempt to repeat the hybridisation at a lower stringency to allow identification of these sequences and quantitation of their similarity to E.coli oriC with more certainty

4.8: Comparison of hybridisation intensities for different probes

In order to see whether further information can be gained by consideration of the quantitative data, I plotted the relative hybridisation intensities for the sum of all homologous bands in each species for each probe used on graphs (see fig 4.6) so that each

species is represented by a point whose coordinates are the relative intensities of hybridisation to probe x and probe y. The data used are given in table 4.6.

If there is a constant relationship between the homology of a particular sequence and the relatedness of the species that carries it to E.coli, when the strength of hybridisation signal for various genes are plotted one against the other, we should expect all points to fall on a line or a simple curve (depending on the exact relationship between hybridisation intensity and sequence similarity). Where points fall off this line, it is an indication that, insofar as the data are reliable, one of the sequences examined is unexpectedly similar, or dissimilar to the E.coli sequence.

If sequences are equally conserved, we will expect relative hybridisation intensities to be equal for different probes in the same species. and thus for the slope of a line drawn through all the points to be 1. In the results shown here, this seems to be most nearly the case for OriC and probe A (see fig 4.6H and table 4.7), suggesting that these sequences have evolved at approximately equal rates. If on the other hand, the rate of divergence of two sequences is different, the slope of the graph will be steeper, or shallower, depending on whether the more conserved sequence is assigned to the ordinate or the abscissa. The results for pCM959 against probeA (4.6F), probe 9.8 against OriC (4.6B), probe 9.8 against probe A (4.6C), pCM959 against probe 9.8 (4.6A) and pPM2000 against OriC (4.6I) would suggest that these sequences evolve at different rates (see table 4.7).

Table 4.6: Relative intensities of hybridisation to probes

Species	no.	oriC	pCM959	9.8	A	pPM2000
		probes				
E.coli	1	1	1	1	1	-
C.freundii	2	0.085	5.25	0.96	0.065	-
E.aerogenes	3	0.255	-	0.51	0.21	0.11
E.cloacae	4	0.094	-	0.48	0.16	1.1
K.pneumoniae	5	0.072	0.27	0.21	-	0.067
S.marcescens	6	0.0051	0.23	0.216	0.006	0.0052
P.alcalifaciens	7	0.012	-	0.05	0	0.016
P.stuartii	8	*	-	0.12	0.0007	-
M.morgani	9	*	-	0.10	0	0.36
P.vulgaris	10	*	0.42	0.06	0.022	0.125
Hafnia alvei	11	0	0.15	0.042	0.037	0.58

-: no data obtained.

*: bands present but too faint to quantify.

0: no detectable homology.

Since no data are available for hybridisation of pPM2000 to *E.coli*, the intensities have been divided by an arbitrary constant to give numbers of convenient dimensions.

Figure 4.6 : Comparison of hybridisation intensity for the probes used in this study

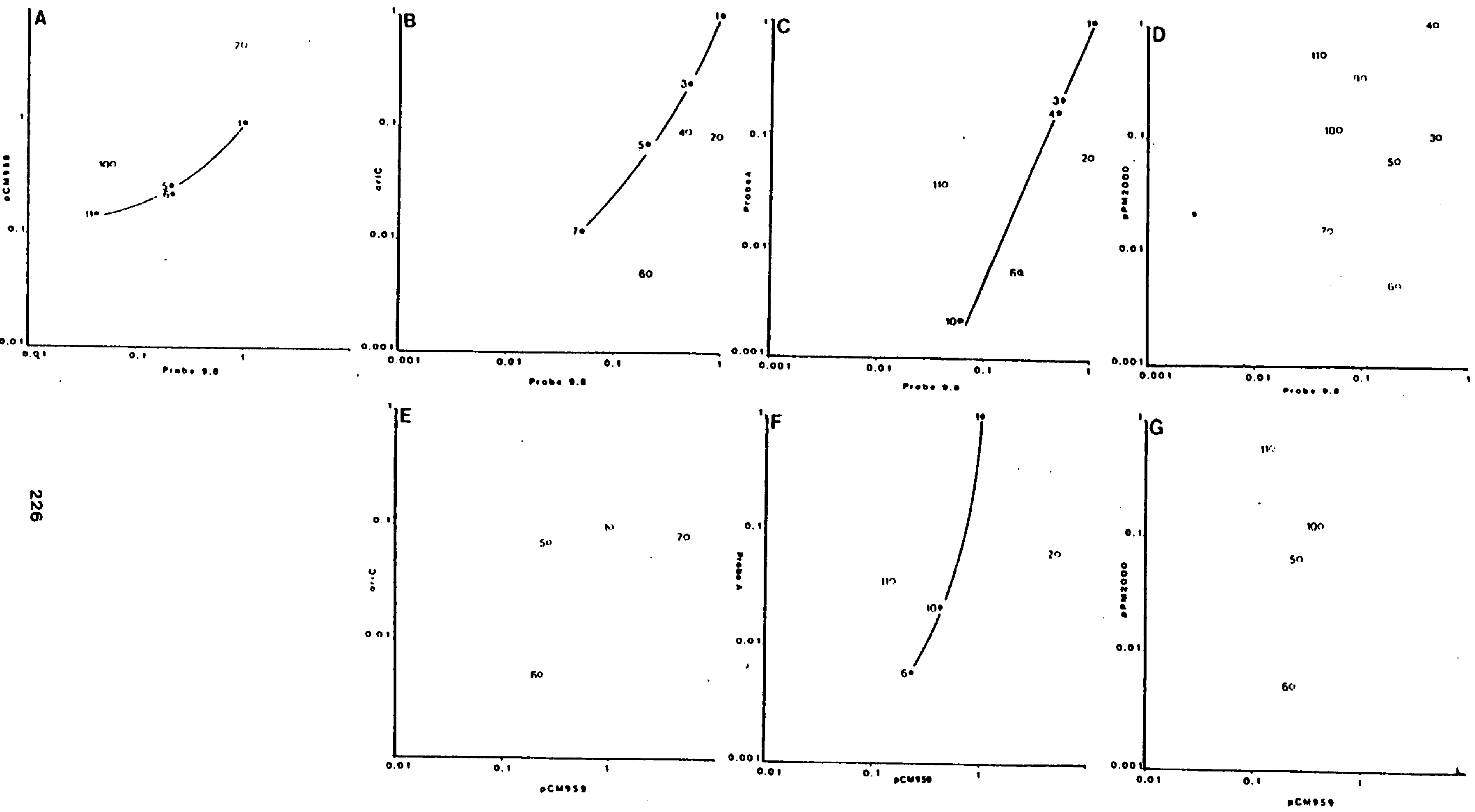
Each species (numbered as below) is represented by coordinates of relative hybridisation intensity to two probes as indicated.

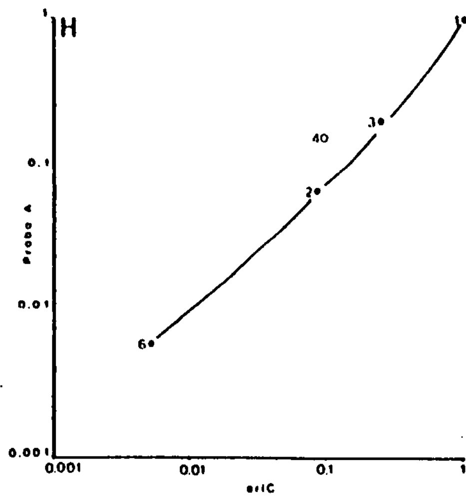
Note that while other intensities have been normalised to *E.coli* = 1, results for pPM2000 have been normalised arbitrarily

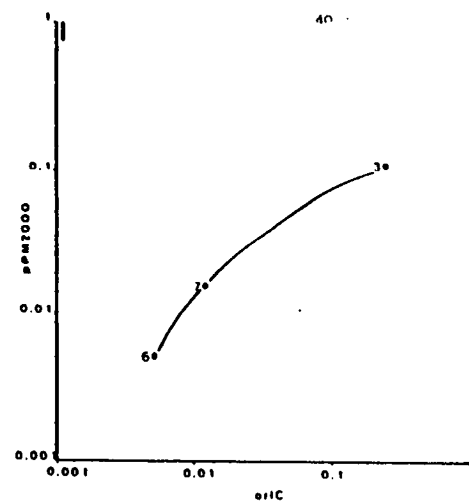
The species are numbered as follows :

1	*E.coli*
2	*Citrobacter freundii*
3	*Enterobacter aerogenes*
4	*Enterobacter cloacae*
5	*Klebsiella pneumoniae*
6	*Serratia marcescens*
7	*Providencia alcalifaciens*
8	*Providencia stuartii*
9	*Morganella morgani*
10	*Proteus vulgaris*
11	*Hafnia alvei*

Figure 4.6




H
1
0.1
0.01
0.001
Probe A
0.001
0.01
0.1
1
oriC
1
3
4O
2
6


I
1
0.1
0.01
0.001
pPM2000
0.001
0.01
0.1
1
oriC
4O
3
2
6

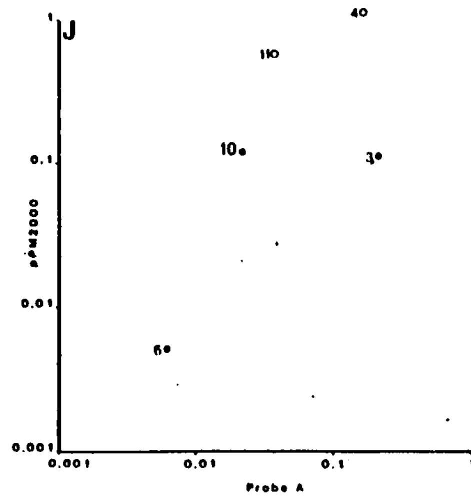

J
1
0.1
0.01
0.001
pPM2000
0.001
0.01
0.1
1
Probe A
4O
11O
10
3
6

Table 4.7 : Slopes of lines from fig 4.6

ordinate	abscissa 9.8	pCM959	OriC	A	pPM2000
probe 9.8	x	0.59	1.45	1.24	-
pCM959	1.49	x	-	3.55	1.32
OriC	0.68	-	x	0.97	-
probeA	0.75	0.28	1.04	x	-
pPM2000	-	0.74	-	-	x

These data are calculated by regression of logx on logy for the points on fig 4.6 represented by filled symbols and falling close to the line drawn on the graph. Due to the effects of regression analysis, the slopes are not quite reciprocal, e.g. probe 9.8 against pCM959 and pCM959 against probe 9.8.

The very large scatter makes the choice of points through which to draw a line to a certain extent arbitrary, so these values must be regarded as very preliminary. In some cases, the errors in estimating the slope may be as much as 2x. However, taking into account this qualification, together with the possibility that differences in hybridisation conditions (such as the degree of fragmentation of the probe) might cause variation in relative hybridisation intensity and thus alter the slopes of the graphs, the relative conservation of the sequences would appear to be:

pCM959 > probe9.8 > OriC = probeA > pPM2000

This differs somewhat from conclusions derived by examining the

bands lit up by various _oriC_ probes, since this suggested that the most conserved DNA is that of the _atp_ operon. I would prefer to leave a choice between these two conclusion until the fragmentary nature of the data for pCM959 can be rectified.

As can be seen, in many cases, results deviate considerably from the normal line. The comparisons for which the data fall on the normal line are:

Ent. aerogenes(3)	OriC, probe 9.8, probe A, pPM2000
C.freundii(2)	probe A, OriC
S.marcescens(6)	probe A, OriC, pPM2000
K.pneumoniae(5)	probe 9.8, OriC
Prov.alcalifaciens(7)	probe 9.8, OriC
Ent. cloacae(4)	probe 9.8, probe A
Prot. vulgaris(10)	probe 9.8, probe A

The results obtained with these "normal" comparisons suggest that the species, in order of relatedness to _E.coli_(1) are:

Ent.aerogenes(3)
Ent.cloacae(4), _K.pneumoniae_(5)
C.freundii(2)
Prot.vulgaris(10)
Prov.alcalifaciens(7)
S.marcescens(6)

The order is obtained by reading the positions occupied by these species from top right to bottom left along the "normal" lines of Fig. 4.6. This order of relatedness differs somewhat from that indicated by the dendrogram of Brenner (1984) shown in fig. 4.1 in that it places Enterobacter closer to E.coli than Citrobacter, and Proteus and Providencia closer than Serratia.

The major "anomalous" results revealed by this analysis are:

Citrobacter freundii (2): the apparent extremely high level of hybridisation of pCM959 to C.freundii DNA is probably erroneous, since it exceeds that obtained in the fully homologous E.coli:E.coli hybridisation, and since OriC gives a "normal" value, when hybridised to the same fragment. An attempt to explain this result would seem premature until its reproducibility can be verified, especially since the result of hybridisation of probe 9.8 to the same fragment does not seem to support the notion that the DNA homologous to pCM959 is duplicated.

More interesting is the very high degree of hybridisation to the 3.25kb putative atp fragment. One might expect that even if this fragment were fully homologous to E.coli DNA, its smaller size (30% of the size of the fully homologous E.coli fragment) would result in its binding only 30% of the radioactivity; although this might not hold true if the probe were not significantly fragmented, so that binding that was quantitative in molar terms would be in excess in terms of micrograms. Whatever the exact physical basis of this result is, it seems that it is this fragment that is responsible for the anomalously high degree of hybridisation of C.freundii DNA to probe 9.8, since if we use the value for the oriC-containing fragment alone, the hybridisation intensity is normal.

Enterobacter cloacae(4): the intensity of hybridisation to OriC is unexpectedly low. The difference from the expected value (0.096 observed, 0.2 expected, see fig. 4.6 B,H), is quite large, though it is possible that this reflects an overestimate of DNA concentration, or

more probably an underestimate of hybridisation intensity, due to the use of an intermediate standard in the calculation of intensity.

Enterobacter cloacae DNA also has an anomalously high homology to the terminus region probe pPM2000. It is possible that this reflects the presence of a multicopy plasmid related to pMB1, the plasmid from which pBR325 was derived (Bolivar,1978) or the presence of amp, tet or cml genes homologous to those in pBR325. This strain is a beta-lactamase producer (Jack & Richmond,1970) which supports this interpretation, although the possibility that this is a chromosomal beta-lactamase is also possible. However, the DNA insert in pPM2000 is from the E.coli replication terminus region where a number of bacteriophage remnants have been found (Kaiser & Murray,1979; Espion et al.,1983). It is possible, therefore that E.coli and Ent. cloacae are lysogenic for two closely related bacteriophages and that this is the cause of the strong hybridisation.

Hafnia alvei (11): Hybridisation to probe A and pPM2000 is much more intense than is expected by comparison with the oriC probes pCM959 and probe 9.8 which, so far as the data can be interpreted, hybridise normally. The result with pPM2000 might well be due to the presence of DNA homologous to pBR325, since examination of chromosomal digests of H.alvei(11) reveals a prominent EcoR1 band (or two prominent HindIII bands) consistent with this strain carrying a high copy number plasmid. This strain was collected in 1943 (Gale and Epps, 1943) before antibiotic-resistance plasmids became common (Watanabe,1963). It seems more probable, therefore, that any DNA homologous to pBR325 is likely to represent DNA from the replication region of the plasmid. The sizes of the HindIII bands lit up by pPM2000 appear to correspond to the sizes of the HindIII fragments of this putative plasmid.

It is not clear whether H.alvei(11) should be regarded as having high homology to probe A or low homology to the oriC region, since there are not yet two independent probes for which the conservation is "normal". Therefore, at present, it can only be suggested that in

H.alvei, at least two out of the three chromosomal regions tested appear to have an anomalous degree of conservation, which may be due to lateral transfer or to differential conservation. Further work using other probes is evidently needed to resolve this difficulty.

Where the faint multiple origins of Prov. alcalifaciens(7) are compared graphically to other sequences, they show the expected degree of homology (see fig 4.6B, 4.6I). In this case, I have used the value for a single fragment, rather than the sum of the values for all the fragments as I did for all the other values plotted. This suggests that the multiple origins are more likely to have arisen by duplication than by lateral transfer and that their rate of sequence evolution is not different from that of other origin sequences, which would indicate that the sequences correspond to functioning origins, rather than "origin pseudogenes" which, being free of functional constraints would evolve at a faster rate.

4.9: Synopsis of results

In summary, these results indicate that:

1) the atp operon (or possible the gidA gene) is the most highly conserved sequence in the origin region.

2) that oriC is more conserved than asnA

3) that in many species, particularly those less related to E.coli, multiple copies of the oriC sequence seem to occur.

4.10: Sequence Similarity

In these pages, I have avoided reference to sequence similarity, except in broad terms, preferring to use "relative intensity" wherever quantitative comparisons were necessary. The reason for this is that the relationship between relative hybridisation intensity and sequence

similarity in Southern hybridisations is not clear. Considering the formula:

$$Tm = \{ 16.6\log[Na+] + 0.41(\%G+C) + 81.5 - 0.65(\%f) - 1.5(\%m) - (500/L) \}$$

Where Tm = Melting temperature of heteroduplex

%f = formamide concentration (v/v)

%m = percentage mismatch

L = Length of duplex

(Marmur & Doty,1962; Bonner et al.,1973; Dove & Davidson,1962; McConaughy et al.,1977; Casey & Davidson,1977)

and the known sequence similarity between E.coli oriC and the replication origins of E.aerogenes, K.pneumoniae and V.harveyi (Zyskind et al.,1983), it would appear that the hybridisations using OriC were conducted at above the Tm of hybrids between these sequences, although hybrids were nonetheless detected. Two explanations might be advanced to account for this; one is to point out that the relationship between Tm and sequence similarity was determined for randomly deaminated bacteriophage T4 DNA (Bonner et al.,1973), and the mismatches between the various oriC sequences are not randomly distributed, but clustered in the spacer regions, so that stability of hybrids will accordingly be higher due to the larger runs of continuous homology. Alternatively, it may be pointed out that the conditions used in these hybridisations: moderately stringent hybridisation (2x SSC, 50% formamide, 37°C) followed by non-stringent washing (2x SSC, 37°C) will ensure that any unstable hybrids formed during the stringent hybridisation will be stable under the washing conditions, and also, that a small amount of low stringency hybridisation between the DNA on the filter and residual probe DNA may occur during the first washing step.

Examination of the parameters which determine the intensity of a hybridisation signal shows that even if the simple relationship between

mismatch and Tm (Tm decreases by 1°C for every 1% mismatch (Casey & Davidson,1977)) can be taken to hold for natural DNA with nonrandomly distributed mismatches, these hybridisation data still cannot afford an estimate of sequence similarity.

An approximate equation for the relative strengths of hybridisation signals in heterologous and homologous reactions has been given by Beltz _et al_.(1983):

$$E_i(t)/E(t) = [1 - (1 - kC_0t)^{-n}][1 - (1 + kC_0t)^{-1}]^{-1}$$

where $E_i(t)$ and $E(t)$ are the extents of the heterologous and the homologous hybridisations respectively at time t, k is the renaturation rate constant for homologous DNA, C_0 is the concentration of single-stranded probe DNA at t=0, and n is the ratio of the rate constant for heterologous renaturation (k_i) and the rate constant for homologous renaturation (k), k_i/k.

This equation can be solved for n or k_i if values of C_0, t, k and $E_i(t)/E(t)$ are available: the term $E_i(t)/E(t)$ is synonymous with relative intensity; t is easily ascertained; and k can be approximately calculated (Wetmur & Davidson,1977; Britten _et al_,1974). It should therefore be possible to calculate the mismatch since a mismatch giving a 10°C reduction in Tm has been shown to reduce the k_i by a factor of 2 (Bonner _et al_.,1973; Casey & Davidson,1977), and the relationship between mismatch and Tm is known (see above). Unfortunately, an essential parameter in the equation to calculate k_i is C_0; a nick-translated probe will have been fragmented in a random and unpredictable fashion (depending on the details of hybridisation conditions, and the molar concentration of hybridising molecules is thus not possible to estimate; furthermore, fragmentation of the probe will affect the value of K_i according to the Wetmur-Davidson equation (Wetmur & Davidson,1968):

$$k = (k_N L^{0.5})/N$$

where L is the length of hybridising DNA, in a similarly unpredictable

fashion.

Also, the relative stability of well-matched and mismatched duplexes may be affected by probe length: an extreme example of this would be if a sequence contained an average of 1 mismatch per 100bp, which would result in a $1^{\circ}C$ decrease in Tm for a long duplex, and the probe were fragmented to an average length of 10bp, in which case, the effect on melting temperature would be negligible.

For this reason, I prefer not to attempt to draw conclusions about amounts of mismatch between these duplexes, and for this reason, it is not possible to say that the atp operon is more highly conserved than oriC solely on the basis of a more rapid drop-off in hybridisation with taxonomic distance when oriC is used as probe than when probe 9.8 is used. Fortunately, the fact that the oriC and atp fragments are distinguishable affords a measure of internal control that allows one to confirm that the difference is not simply due to differences in hybridisation conditions. If absolute estimates of sequence similarity are required, it will be necessary to use probes of more well defined composition, such as end-labelled restriction fragments, or alternatively to attempt to determine melting profiles.

4.11: Discussion of the Method

The results presented here demonstrate the possibility of obtaining useful quantitative data from Southern hybridisations. A number of improvements to the approach already described here could be suggested:

1) Probes: apart from the obvious advantages of having further probes on which to draw for the construction of "normal curves" when plotting hybridisation data, it is clear that these results would have been more readily comprehensible if the probes had been monofunctional: if the atp, oriC, and asn DNA had been on separate probes. Where probe DNA was from λ clones, I chose to purify the probe DNA away from λ sequences. This may well be a

counterproductive refinement, since the presence of λ DNA in equimolar concentration with the sequence under study in the probe would have furnished a valuable internal standard, since in E.coli and a number of related species, there is chromosomal homology to varying degrees with many λ sequences (Anilionis and Riley,1980). If the relative intensities of λ-homologous bands remained constant between experiments (or varied in some simple manner), it would be possible to place more faith in inferences about sequence similarity drawn from relative hybridisation intensities. The disadvantage with using a probe containing λ DNA is that bands of interest may be obscured by λ-homologous bands.

2) DNA: No attempt was made to determine the size of the undigested chromosomal DNA used here but examination of the undigested DNA on gels showed that the size was approximately 40kb. This would mean that the 30-40% of a fragment of the size of the 15-19kb K.pneumoniae oriC fragment would be found in fragments with an undefined sheared end. Clearly it is desirable to know the size distribution of the DNA used and correct the values obtained for large fragments, where necessary.

3) Gels: In view of the inaccuracies that may result in the use of intermediate standards, it would be desirable to include all digests on a single filter, or at least to include a fully homologous standard in all gels.

4) Processing the data: The data presented here were processed by hand and this is time-consuming. Any future analysis using this method would be usefully preceded by devising a computer program to handle the data.

5) Reproducibility: In no case have these results been replicated. The accuracy of the data that this method produces still therefore needs to be determined.

Given these stipulations, the method has a number of advantages over other quantitative hybridisation procedures:

1) Since the signal is seen as a distinct band, background caused by non-specific binding of probe to the filter is not a serious problem, since this is automatically corrected for.

2) Fragment sizes can be determined and may give valuable clues as to the distribution of homology within the sequences under study.

Chapter5

On the Isolation of Bacterial Origins of Replication

Several approaches to the cloning of bacterial origins of replication relying on direct selection of either the origin itself (Lovett and Helinski,1976; Yasuda & Hirota,1977; Diaz & Pritchard,1978; Harding *et al*.,1982) or a closely linked marker (von Meyenburg *et al*.,1978a; Miki *et al*.,1978) have been devised. There are two approaches to direct selection, although these do not really differ in their basis: The first involves the ligation of DNA known to contain a replication origin to a purified DNA fragment containing an antibiotic resistance marker, but intrinsically incapable of autonomous replication, and selection for this marker in transformed cells which will contain the *in vitro* constructed replicon bearing the selected replication origin (Hirota *et al*.,1977; Meijer *et al*.,1978; Diaz & Pritchard,1978). An alternative method is to use, in place of the purified antibiotic resistance fragment, a ColE1-derived plasmid cloning vector whose replication is disabled by the presence of a *polA* mutation in the transformation host, and to select for antibiotic-resistant transformants as before (Harding *et al*.,1982).

In the experiments described below, I chose to use the latter approach for the following reasons: firstly it allows the possibility of amplifying the resulting plasmids in a *polA*+ background (a useful property, since plasmids whose replication is driven by chromosomal replication origins are characteristically of low copy number and unstable which renders them difficult to purify in useful quantities); secondly, it obviates the need for purifying the antibiotic resistance fragment away from the plasmid origin of replication; and finally, it allows assessment of the efficiency of the ligation reaction by transforming the ligation mix into a *polA*+ host, which permits discrimination between a negative result due to failure to isolate a functional origin from a statistically significant number of recombinant clones and a negative result due to a failed ligation.

I attempted to isolate ori-plasmids from libraries of EcoR1-digested chromosomal DNA ligated into the cat gene of pBR325 and transformed into the polA,hsdR,hsdM+ transformation host ND13, a derivative of ED8641 constructed specially for the purpose. Positive results were first obtained with Alcaligenes faecalis, and since this species is from a different family than E.coli (Alcaligenes is not at present assigned to any Family, but it appears to belong to rRNA group II of the Pseudomonadaceae (Kersters & De Ley,1984)), it was felt that is a possible very distantly related chromosomal replication origin, the putative A.faecalis oriC would make an interesting comparison with E.coli oriC.

5.1. Screening of an Alcaligenes faecalis library for origins of replication

When an EcoR1 library of A.faecalis DNA in pBR325 was transformed into ND13 (polA) 9 colonies resistant to 5μg/ml of tetracycline were isolated. These were tested for plasmid-born antibiotic resistance markers and stability by streaking out from single colonies growing on LB+5μg/ml tet, growing to single colonies overnight and screening for antibiotic resistance by replica plating onto LB + 5μg/ml tet, 5μg/ml amp or 20μg/ml cml. (20μg/ml cml was found to somewhat inhibit the growth of all the colonies tested and so in later experiments, on which some of the data of table 5.1 are based, the level of this antibiotic was reduced to 10μg/ml. Retention of the polA mutation was confirmed by testing for sensitivity to ultraviolet irradiation as described in Materials and Methods. The presence of pBR325 DNA was tested by the colony hybridisation method of Grunstein and Hogness, using ^{32}P-labelled nick translated pBR325 as probe.The results of these tests are summarised in table 5.1.

On the basis of this table, 6 classes of strain can be distinguished. ND20 and ND17 carry pBR325, and are presumed to contain recombinant plasmids capable of autonomous replication in the polA background, and therefore to represent cloned origins of replication. The plasmids they

Table 5.1. Properties of Tet^R strains obtained after transformation of A.faecalis library

clone no	strain designation	antibiotic resistance	stability	pBR325 DNA	UV sensitivity
1	ND20	amp,tet,cml	unstable	+	s
6	ND17	amp,tet	unstable	+	s
2	-	amp,tet,cml	unstable	NT	s
5	ND22	amp,tet,cml	unstable	-	s
7	ND24	amp,tet,cml	unstable	-	s
3	ND21	tet	unstable	-	s
10	-	tet	unstable	NT	NT
4	ND19	amp,tet	unstable	-	s
8	ND18	amp,tet,cml	stable	-	s

carry are called pND3 (ND20) and pND4 (ND17). The remaining 4 classes, containing 7 isolates, lack pBR325 homology and are therefore presumed to be E.coli chromosomal antibiotic resistance mutants. These are assigned designations as in table 5.2.

Instability of both mutant and plasmid-carrying strains was further investigated by plating overnight broth cultures, grown up with selection for Tet^R, to single colonies on LB and replica plating. It was possible to quantitatively score both for complete antibiotic sensitivity and for colonies with antibiotic-sensitive sectors resulting from segregation of antibiotic resistance during growth on L-Broth. Results are summarised in table 5.3.

ND17 gave rise to Tet^R,Amp^S (5%) and Tet^S,Amp^R (2.2%) colonies, which suggests that the plasmid pND4 is prone to instability by deletion.

The three cultures of ND21 tested were so different as to call into doubt their common origin. One culture was, like the parent culture, Tet^R,Cml^S,Amp^S but two other cultures were respectively Tet^R,Amp^R,Cml^R and Tet^R,Amp^R,Cml^S. Strains of this class (TCR3,TCR10) were not studied further.

The stability of strains ND17 (pND4), ND20 (pND3), ND19 (TAR1), ND22 (MAR5), and ND24 (MAR7), was further investigated. The rate of loss of antibiotic resistance was determined by growing cultures in antibiotic-free medium (LB) and plating for viable cells and antibiotic resistance at appropriate intervals. Figure 5.1 shows graphs of the curing kinetics of ND17, ND19, and ND22, and a summary of the curing rates is given in table 5.4. The curing rate of a plasmid can also be used to estimate its copy number if its segregation is random (Meacock & Cohen,1982). The copy numbers of randomly segregating plasmids that would give the curing rates observed are also given in table 5.4.

A number of points of interest emerge from these data. Firstly, the putative plasmid-carrying strains ND17 and ND20 are the most unstable. ND17 shows the exponential curing, kinetics expected of

Table 5.2 Antibiotic Resistance Mutants

clone no.	strain name	designation of mutation
2	-	MAR2
5	ND22	MAR5
7	ND24	MAR7
3	ND21	TCR3
10	-	TCR10
4	ND19	TAR4
8	ND18	SAR8

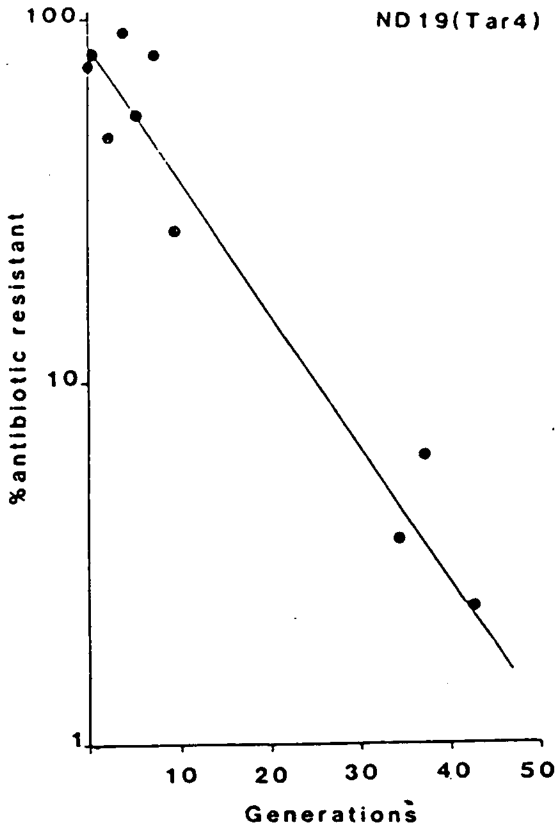
The names of the mutations signify Multiple Antibiotic Resistance (MAR), Tetracycline Resistance (TCR), Tetracycline and Ampicillin Resistance (TAR) and Stable Antibiotic Resistance (SAR).

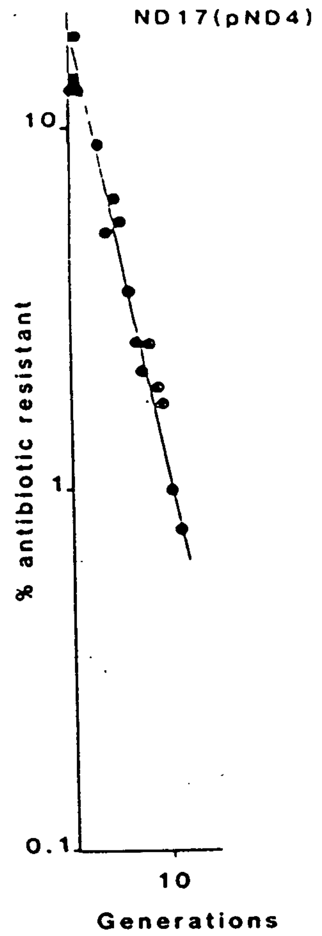
Table 5.3: Stability of antibiotic resistance

strain	resistance factor	% sectored	% antibiotic sensitive
ND20	pND3	50%	<0.24%
ND17	pND4	27%	66%
ND19	TAR4	16%	1%
ND22	MAR5	42%	1.2%
ND24	MAR7	51%	<0.4%
ND21	TCR3	30%	1.5%
ND18	SAR8	<0.08%	<0.08%

Results are averages from 4-9 different overnight cultures. Sectoring was scored on the cml plate wherever possible and if not, on the tet plate as it was more easily scored on these plates. Complete antibiotic resistance of a colony was recorded if it was sensitive to all 3 antibiotics.

Figure 5.1





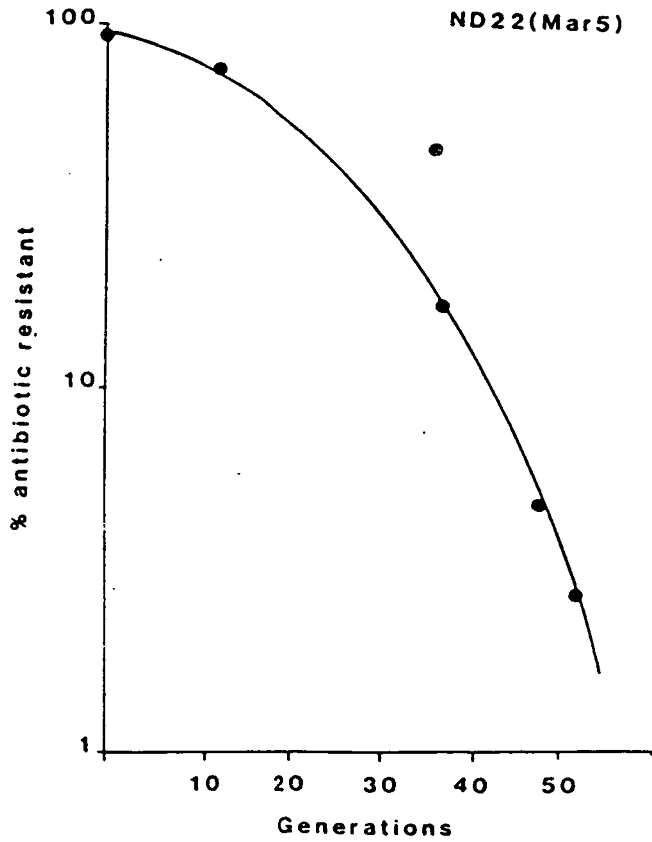


Figure 5.1: Curing kinetics of pND4 and reversion of TAR4 and MAR5

Cultures were maintained in exponential phase in LB and periodic samples diluted and plated on LB and LB+5μg/ml tetracycline.

Table 5.4 Curing rate (% loss of antibiotic resistance/cell generation)

Strain	curing rate	estimated "copy number"
ND17 (pND4)	15%, 23% (two independent estimates)	3.7, 3.1
ND20 (pND3)	35%	2.5
ND19 (TAR4)	8%	4.6
ND22 (MAR5)	2%*	
ND24 (MAR7)	3%	6.1

* See fig.5.1 and text for comments on kinetics

Curing rate was calculated according to the formula :

$C = 1 - (a/A)^{1/N} \times 100\%$ per cell generation

where N is the number of cell generations elapsed and A and a are the initial and final proportions of antibiotic-resistant cells respectively. Values for ND20 and ND24 are based on two measurements only.

Copy number was estimated according to according to the formula:

$$P = 2(0.5)^N$$

where P is the probability of either daughter cell failing to receive a plasmid at cell division, and N is the plasmid copy number at cell division. P is equal to the curing rate expressed as a frequency rather than as a percentage.

unstable plasmids (Novick et al.,1975). The TAR and MAR strains are markedly more stable. MAR5 is more stable than TAR4 and shows a downward deviation from exponential kinetics. This would be explicable if the cured cells have a growth advantage over the antibiotic-resistant cells, so that as cured cells come to predominate, their faster growth rate causes an apparent acceleration of the apparent rate of curing which is actually due to takeover by these cells.

ND19 TAR4 is intermediate in stability between MAR and the plasmids and appears to show exponential curing kinetics.

These results largely agree with the data of table 5.3, with the exception of ND20 (pND3) which, according to those data is comparable in stability to the MAR strains. Pending further investigation of this anomaly, it seems sensible to take the results of the curing kinetics as the true value and to regard the result in table 5.3 as erroneous.

5.2: Preparation of plasmid DNA

I attempted to isolate plasmid DNA from ND17 (pND4), ND19 (TAR4), and ND24 (MAR4), by 3 different methods: the small scale alkaline lysis method of Birnboim and Doly, the large scale alkaline denaturation procedure of Hansen and Olsen, and a neutral lysis procedure. None of these procedures afforded plasmid DNA that was detectable either by agarose gel electrophoresis or by fluorescence in EtBr/CsCl gradients, despite many attempts.

In two gradients, prepared from lysates of ND24 (MAR7) and ND19 (TAR4) by the large scale alkaline lysis procedure, it was possible to detect a sharp and very faint band below the chromosomal DNA in the position where plasmid DNA was expected to be found. However, when this was collected and digested with EcoR1, it was found to contain

only chromosomal DNA, and it was unable to transform polA or polA+ strains to Tet^R. It is conceivable that this band actually represents supercoiled chromosomal since the method employed has been found to be specially suited to the isolation of very large plasmids (Meulien,1982), and there is in principle no reason why it should not be extendable to entire bacterial chromosomes. Should this prove the case, this method may well prove useful for the isolation and study of intact bacterial chromosomes.

The failure of MAR and TAR strains ND24 and ND19 to yield fractions able to transform either a polA or a polA+ host is further evidence that these strains are not plasmid transformants at all, but rather they are chromosomal mutations. Although these strains lack any sequence homology to the cloning vector pBR325, the possibility that an unrelated Tet^R,Amp^R,Cml^R determinant from *Alcaligenes* is responsible for their phenotype cannot at present be rigorously eliminated, although the failure of all efforts to obtain transforming DNA from these strains eliminates the possibility that such a factor could exist as a plasmid.

In the case of ND17 (pND4), however, when caesium chloride gradients prepared by alkaline or neutral lysis procedures were fractionated and the fractions transformed into polA and polA+ recipients a peak of transforming activity separate from the viscous chromosomal DNA fraction was observed. The measured density of this fraction was 1.60 g/cm^3. However, only the polA+ host could be transformed with this fraction. Transformants were, like ND17, Tet^R,Amp^R,Cml^S and carried a plasmid which on digestion with EcoR1 yielded fragments of 6kb and 5.8kb and thus is apparently derived by insertion of a 5.8kb EcoR1 fragment into pBR325. However, even when these transformants were used as a source of transforming DNA, no transformants of a polA host could be obtained, although the quantity of DNA was physically detectable (0.05 µg).

A fractionated gradient prepared on a different culture of ND17 yielded two transformants which gave a single band of approximately 8kb on digestion with EcoR1, which I will refer to below as pND48.

Retransformation of pND4 and selection on either 50µg/ml or 10µg/ml of ampicillin allowed recovery of the same plasmid plus three distinguishable classes of deletion derivative: pND41, pND44, and pND46, which gave a single band of 1.65kb on digestion with EcoR1; pND42, which gave bands of 7.7 and 1.65kb; and pND43, which gave bands of 3.7 and 1.65kb. These derivative plasmids were all isolated on 10µg/ml ampicillin, although they could be subcultured on 50µg/ml ampicillin.

Two features of this collection of spontaneous deletion derivatives are noticeable. The first is the presence of a common 1.65kb band. Assuming that pND4 is the parent of these plasmids, it is difficult to see how both plasmids consisting of only a 1.65kb molecule with a single EcoR1 site, such as pND41, and plasmids consisting of this fragment plus a larger EcoR1 fragment could arise. The two fragments may actually be parts of different molecules, since any simple deletion event generating, as in pND42, a fragment larger than either of the two EcoR1 fragments of pND4, must necessarily delete one of the EcoR1 sites, so that a second fragment would be precluded. It seems more likely that "pND42" actually consists of two plasmids, a 1.65kb plasmid like pND41, and a 7.7kb plasmid. This would account for the second noticeable feature; the apparent similarity between pND48 and the large band of pND42. This would suggest that pND4 is prone to break down in a predictable fashion and that in the "pND42" and "pND43" strains, the mixed plasmid population resulting from this has been detected. The antibiotic resistances of these deletion derivatives has yet to be investigated; but it seems highly unlikely that, considering its very small size, pND41 contains any more DNA than the replication region and ampicillin resistance gene of pBR325. The very small size of pND41 is surprising and suggests that the origin of these plasmids may be more complex than suggested here. The distance between the EcoR1 site and the replication origin of pBR325 is more than 2.2kb, and so a single deletion could not generate a plasmid containing <u>ori</u>, <u>amp</u> and the EcoR1 site.

It seems likely that deletions of this sort are responsible for the small percentage of Amp^s,Tet^R and Amp^R,Tet^s derivatives noticed during

experiments to assess the stability of antibiotic resistance in ND17.

By optimising the method of Birnboim *et al*. it proved possible to isolate three pND3 transformants of the *polA*+ parent of ND13, ND11, from a plasmid preparation on ND20. Digestion of these plasmids with EcoR1 resulted in a single band of 4.6kb. The culture from which the DNA for these transformants was obtained was resistant to ampicillin and tetracycline, but the phenotype of the transformants has yet to be investigated. If these plasmids are the wild-type pND3, then it would be surprising if, in view of their single EcoR1 site and small size, they contained any insert DNA.

Thus, although plasmid DNA can be isolated from ND17 and though this strain remains sensitive to UV irradiation, the plasmid isolated from this strain is unable to retransform a *polA* host. Therefore I am unable to confirm that pND4 carries an origin of replication.

Two explanations might be advanced for these results. One is that ND17 is a partial *polA*+ revertant, or carries a weak amber suppressor of the *polA21*(Am) mutation of ND13 (with the same phenotypic consequences), such that low-level replication of pBR325 derivatives can occur, but UV-resistance is not restored. That this is probably not the case is demonstrated by the fact that a tetracycline-sensitive derivative of ND17 which had presumably been cured of pND4 could not be retransformed with pBR325. Another host mutation that would allow replication of a *polA*-dependent plasmid is *pir* (*polA*-independent replication) (Kogoma *et al*.,1984). This is an allele of *rnh* and is actually a manifestation of the Das/Sdr complex of phenotypes. Although the failure of pBR325 to retransform ND17 argues against this explanation in the same way that it does against the hypothesis of a weak *polA*+ revertant, the failure to isolated plasmid DNA might be explicable since *polA*-dependent plasmids isolated from *pir* strains are found to be highly concatomeric.

Alternatively, it might be suggested that pND4 does not replicate autonomously at all in ND17, but instead exists as a highly unstable

chromosomal insertion that cures readily by looping out to form a non-replicating supercoiled circle, which is presumably the source of transforming activity in the non-chromosomal fraction.

This latter hypothesis suffers from the objection:"If it was possible to obtain pND4 transformants when this plasmid was present in the minute molar amounts typical of a ligation mix, how is it that when the same plasmid is amplified many thousand fold and transformed into the same host, no Tet^R,Amp^R transformants can be obtained?" Such might be the case were pND4 merely the passive target sequence for a host-specified cointegrate formation due, for instance to one of the many chromosomal insertion elements.

Since the cured derivative of ND17 does not retransform with pBR325, it clearly does not have a heritable propensity to form chromosomal cointegrates. One could account for the apparent readiness with which deletion derivatives of pND4 arise in polA+ strains, by suggesting that this plasmid is predisposed to illegitimate recombination, but this would conflict with the idea of this plasmid having only a passive role in the formation of cointegrates with the chromosome.

5.3. Comparison with other origin cloning experiments

By way of comparison with these results, the data obtained from attempts to clone origins of replication from Morganella morgani, Vibrio natriegens and Escherichia coli, is presented below:

5.3.1. Vibrio natriegens

The genus Vibrio is closely related to the Enterobacteriaceae (Baumann and Baumann,1977) and a 6Kb SalI fragment containing the putative chromosomal origin of replication of V.harveyi is able to replicate in E.coli and shows 70% homology to E.coli oriC (Zyskind et al.,1983).

I attempted to isolate transformants of ND13(polA) resistant to 20μg/ml of chloramphenicol from a HindIII library of V.natriegens DNA in

pBR325 containing an estimated 5000 recombinants, but no transformants were obtained. I conclude that V.natriegens either lacks an origin of replication which can function in E.coli (a notion which is in agreement with the lack of any detectable homology between E.coli oriC and V.natriegens chromosomal DNA, see Fig 4.4), or if such an origin is present, it is cleaved by HindIII. E.coli oriC does contain a HindIII site within a variable region of the minimal origin sequence, but this is not true of the V.harveyi ori sequence, nor of the consensus sequence. V.natriegens is not, however, particularly closely related to V.harveyi (Baumann & Baumann, 1977), so there is no particular reason to expect the sequences of their replication origins to be more related than those of, for instance, Erwinia carotovora and E.coli.

Alternatively, one might suggest that the oriC-containing HindIII fragment of V.natriegens is very large, and that the combined effects of the preference of ligation reactions for small fragments, the lower efficiency of transformation of large plasmids and the increased likelihood of the fragment being truncated by shearing all militated against the recovery of Ori+ recombinants.

5.3.2. Morganella morgani.

Morganella morgani DNA shows very weak homology to the minimal oriC sequence. This is present in three EcoR1 bands of 5.7, 7.8 and 10.7kb (see table 4.3). When an EcoR1 library of M.morgani DNA in pBR325, containing an estimated 1000 recombinants was transformed into E.coli ND13 and transformants resistant to 10μg/ml of tetracycline were selected, a single Tet^R colony, ND15 arose. In its stability and range of antibiotic resistances, this strain resembles the MAR mutants, isolated during attempts to clone a replication origin from A.faecalis, but it is distinguishable from these on the basis of its disturbed cell morphology. It is assigned the designation MAR14 and has the following properties:

1) Resistance to ampicillin, chloramphenicol, and tetracycline: the concentration of these antibiotics in plates required to reduce the efficiency of plating to 50% ($E.O.P._{50}$) was determined for this

strain and for its parent ND13 (Table 5.5).

2) Antibiotic resistance is unstable: a few percent of Amp^S, Tet^S being found even in cultures grown under selection for antibiotic resistance, as was the case with the MAR strains ND22 and ND24.

3) Colony morphology is unstable and this is associated with changes in cell size and shape: Colony morphology variation was also encountered in other MAR strains and in TAR, TCR and pND4 and pND3 carrying strains.

4) Cell morphology is disturbed: cell morphology of ND15 was highly dependent on growth conditions. Old stationary phase cultures were indistinguishable from similar cultures of the parent strain ND13, while actively growing cultures were typically filamentous. Severe filamentation appeared to be associated with a colony morphology variant which gave colonies which were much denser in appearance than was usual for this strain. Such variants were also slow growing in liquid culture. Cultures of such a variant were found to have a very broad cell size distribution while cultures of the 'normal' colony former had a narrower size distribution, though still much broader than that of the parent strain, ND13. The cells were, on average, larger than those of ND13 (see Fig. 5.2). This suggests that MAR14 might be a lon mutation, since lon mutants, which are defective in a major protease (Charette et al.,1981), show enhanced resistance to antibiotics (Reeve,1968; Reeve & Suttie,1968), and have enlarged cells.

ND15 is not a polA+ revertant, since it is as UV-sensitive as its parent ND13 and antibiotic-sensitive derivatives of it cannot be transformed with the polA-dependent plasmid pACYC184. Colony hybridisation shows that it carries no sequences homologous to pBR325.

Table 5.5 E.O.P.$_{50}$ of chloramphenicol, tetracycline, and ampicillin in LB agar for ND13 and ND15

	tetracycline	ampicillin	chloramphenicol
ND13	1-2.5μg/ml	<2μg/ml	4-6μg/ml
ND15	10-20μg/ml	6μg/ml	50μg/ml

5.3.3. E.coli

EcoR1 libraries in pBR325 were prepared from both whole E.coli DNA from strain C600 and from λEH11, which carries the 9.8kb oriC containing EcoR1 fragment (see fig 4.1).

2 Amp^R colonies obtained from transforming the chromosomal DNA library into the polA- strain ND12 (the Tet^R parent of ND13) were used to prepare plasmid DNA by the method of Birnboim and Doly and were shown to be able to retransform ND12 to Amp^R.

Since I was unable to visualise plasmid DNA in the polA transformants, I used P1 transduction to test whether the plasmids were free in the cytoplasm or integrated at oriC or some other chromosomal location. Plasmids carrying oriC, and particularly those which carry the whole of the oriC containing EcoR1 fragment, integrate very readily into the chromosome by a recA-independent recombination process (Masters et al.,1978), which affords stability to otherwise highly unstable plasmids and relieves the reduced growth rate and disturbed cell morphology that strains carrying such plasmids otherwise suffer. Thus, cultures of strains carrying large oriC plasmids contain a large proportion of cells in which the plasmid is integrated at oriC and is thus cotransducible to the flanking marker asnA. Table 5.6 shows the cotransduction of plasmid markers with asnA.

The pND01-carrying strain does not cotransduce antibiotic resistance and asnA and thus the plasmid either is not an oriC plasmid, has not yet succeeded in integrating in this strain, or is integrated at another locus. It is not known whether ND12 (in which pND01 was originally isolated) and R2D2 and its derivative ND14 carry the rac prophage. If not, then the replication of oriJ would not be repressed in these strains, and pND01 might replicate via oriJ. Both the pND02 carrying strains cotransduce Amp^R and asnA, indicating that the plasmid is integrated close to asnA and therefore oriC. The cotransduction frequency determined for Asn+ and Amp^R is close to that predicted from the known distance between asnA and oriC plus the length of the

Table 5.6 Frequency of Amp^R,Tet^R cotransductants among Asn+ transductants from donor strains carrying putative pBR325oriC plasmids pND01 and pND02

recipient	relevant markers	donors		
		ND12pND01	ND12pND02(a)	ND12pND02(b)
R2D2	asnA,asnB,polA+	0% (47)	53% (15)	26% (15)
ND14	asnA,asnB,polA-	0% (74)	35% (64)	59% (44)

Cotransduction was assessed as the proportion of Asn+ transductants scored as Amp^R,Tet^R. The bracketted number is the total number of Asn+ transductants screened.

predicted plasmid (16kb). The physical distance between oriC and asnA is approximately 2kb so that the genetic distance between Amp^R and asnA is expected to be equivalent to 16 + 2kb, or 0.4 mins; corresponding to a cotransduction frequency of 48%. The cotransduction of pND02 markers with asn is strong, though not conclusive, evidence that this plasmid carries oriC.

The transduction of a polA+ host to Amp^R is rather unexpected as plasmids carrying the 9.8kb oriC EcoR1 fragment are usually found to be highly deleterious to cell growth even in their low oriC-dependent copy number of 2-4 (Messer et al.,1978). polA+ strains transduced with pND02 are expected to harbour the plasmid as a high copy number replicon and thus incur even more severe effects on growth. One explanation of this apparent discrepancy would be to suggest that in pND02 the strong chloramphenicol promoter in pBR325 is reading into the atp operon whose over-expression is considered to be responsible for most of the deleterious effects of oriC plasmids (von Meyenburg et al.,1984). In one of the possible orientations, the chloramphenicol promoter will read in the opposite direction to the atp promoter and the colliding transcription resulting from this would be expected to interfere with atp operon expression.

Four ND13 transformants obtained by ligation of EcoR1-digested λEH11 and pBR325 were also investigated. When first isolated, these were seen to be filamentous as is usually the case with strains carrying plasmids with the EcoR1 oriC fragment. However, on subsequent examination they were found to be morphologically normal and stable, which is typical of a strain in which an oriC plasmid is integrated (Masters,1978).

Small-scale plasmid preparations from these strains digested with EcoR1 gave no bands on an agarose gel, nor could a CCC band or a fraction yielding transforming activity be detected on a caesium chloride gradient prepared from a litre of stationary phase cells. Although the kinetics of curing of these plasmids has not been investigated, when cells were grown under selection for antibiotic resistance and subsequently plated to single colonies on LB, occasional

antibiotic-sensitive colonies arose at a frequency of approximately 0.4%. This indicates that antibiotic resistance is more stable in these strains than in the MAR strains, but less so than in the SAR strain. Furthermore, the SAR strain had enlarged cells, a phenotype that disappeared from these strains on subculturing. These observations seem to preclude the possibility that these ND13 derivatives owe their antibiotic resistance to chromosomal mutations similar to those suggested for the MAR and SAR strains.

Another possible explanation for the phenotype of these strains, other than that they carry authentic oriC plasmids (albeit one which would not explain their initial filamentous phenotype) is that they are lysogenic for lambda-pBR325 hybrids created by substituting the oriC insert in λEH11 with pBR325. If this were the case, these strains would be expected to express the immunity of λEH11, imm^{21}. Two of the four transformants were tested and found to be sensitive to $\lambda imm^{21}cI$, indicating that these strains were not lysogens.

Thus it seems likely that these clones (designated ND160 - ND163) carry integrated pBR325-oriC hybrids, though the linkage of plasmid markers to markers in the oriC region remains to be demonstrated.

5.4. Cell Morphology

It has been noted that many of the larger oriC plasmids confer a morphology defect known as het (heterogeneous cell size, von Meyenburg et al.,1978) and that some of the presumed mutant strains, notably ND15 (MAR14) and ND18 (SAR8), also display disturbed morphology.

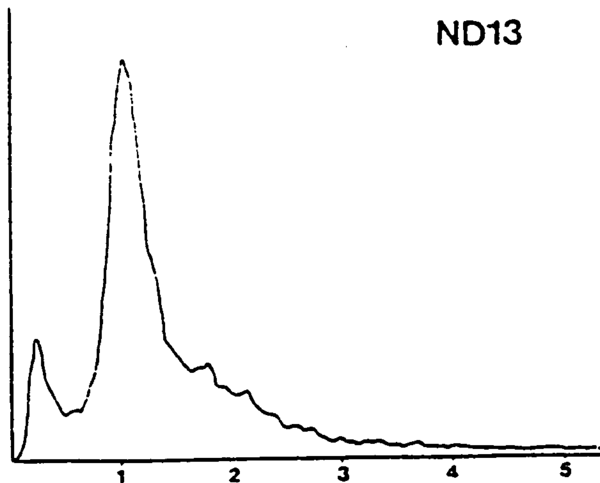
I was therefore interested to know how many of the various tetracycline-resistant isolates shared these disturbed morphology phenotypes, especially since all but ND18 and ND160, ND161, ND162 and ND163 resemble ND15 in displaying frequent two-phase variation in colony morphology.

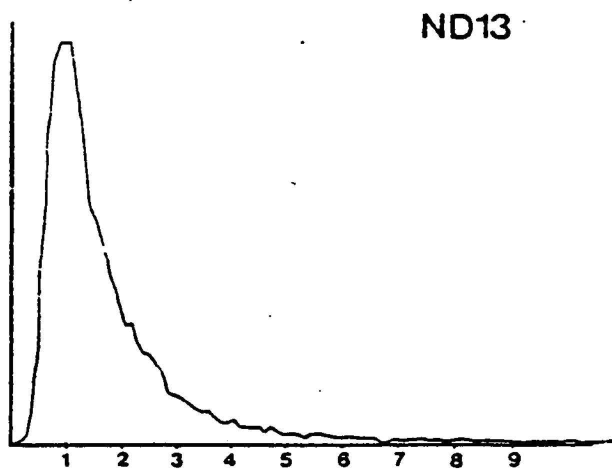
Cultures in LB+5μg/ml tet were examined by light microscopy and cell sizes were determined using a Coulter counter. Only ND15, ND18 and the pND4-carrying strain ND17 were found to have a disturbed morphology. ND15 has been discussed above. The cell size distributions are shown in Fig. 5.2.

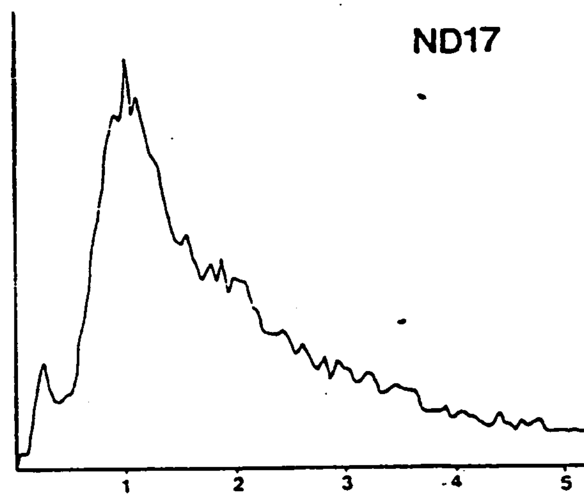
ND17 cultures showed an heterogeneous cell morphology which includes minicells and filamentous cells of varying length; which resulted in a noticeable skew in the cell size distribution relative to the parent strain, ND13. Minicells could not be distinguished from debris by the Coulter counter. Filamentous cells of ND17 were often seen to contain refractile inclusions. Since the population is composed mostly of normal cells, and since a substantial proportion of plasmid-free cells is found even in cultures grown under selection, it is not clear whether the abnormal cells are the consequence of the presence of pND4, or its absence; that is to say, a Het-like phenotype, or the consequences of poisoning of Tet^S cells by tetracycline. However, the fact that a similar morphology was observed in polA+ cells carrying pND4 in high copy suggests that the former is more likely to be the case.

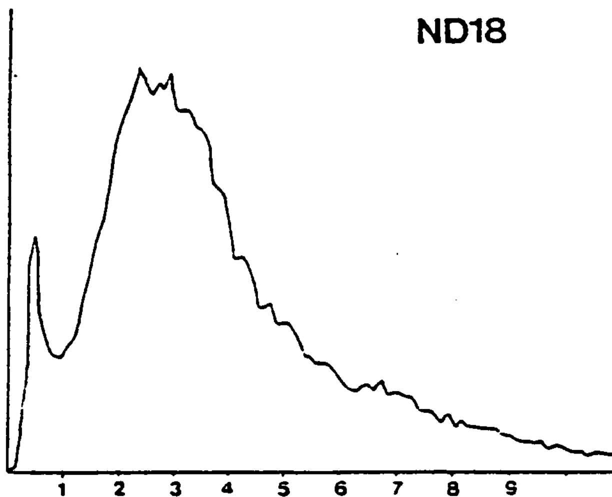
Comparison of the cell size distributions of ND18 and its polA,Tet^S parent ND13 shows that ND18 had an elevated cell volume (modal volume 3 - 4 fold increased over ND13), and this was reflected in the appearance of its cells. A preponderance of elongated cells, up to 8X the normal length was observed, though unit length cells were also seen. The phenotype of ND18 would be most simply explained by an envelope mutation affecting both cell division and permeability to antibiotics. The MAR14 phenotype might also have a similar basis; however, these mutants also resemble lon strains of E.coli, whose antibiotic resistance and excessive cell size is due to the absence of a major protease (Charette et al.,1981). In the latter case, resistance to the protein synthesis inhibitors chloramphenicol and tetracycline would presumably be due to an increased survival time of proteins in the cell and thus a reduced need for ongoing protein synthesis, while increased ampicillin resistance might be explained by a reduced frequency of cell division,

Figure 5.2









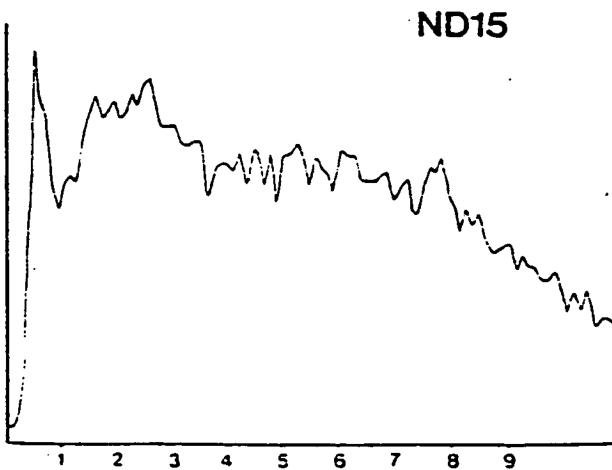


Figure 5.2: Cell size distributions

Cell size distributions of log-phase cultures of ND13 (*polA*), ND17 (*polA*,pND4), ND15 (*polA*,MAR14) and ND18 (*polA*,TAR4) were determined using the Coulter Channeliser. The scale along the axis is in multiples of the modal cell volume of ND13. Distributions on the right hand side of the figure are determined with 2-fold lower amplification setting than those on the left, to accommodate the broader size distributions of ND15 and ND18.

the most ampicillin-sensitive step in the cell cycle (Lederberg,1956; Mathison,1968).

5.5. Discussion

It seems plausible that the MAR mutations in ND22 and ND24 are alleles of the marA gene (George & Levy,1983a;1983b). Mutations at this locus, which maps at 34' on the E.coli genetic map, give concomitant resistance to low levels of tetracycline, chloramphenicol, and ampicillin, as well as some other antibiotics. Although tetracycline resistance mutations at the marA locus are recessive, they are not null mutants; such mutants have been isolated by Tn5 insertion mutagenesis, and gave increased sensitivity to tetracycline and chloramphenicol. George and Levy (1983a) demonstrated that in addition to tetracycline, chloramphenicol, and ampicillin resistance, their mutants were also resistant to minocycline (a lipophilic tetracycline), penicillin G and cephalothin (β-lactams), puromycin, and rifampicin, and showed augmented resistance to nalidixic acid. Resistance to the aminoglycosides streptomycin and kanamycin was not affected, and in common with other tetracyline-resistant strains, their mutants were hypersensitive to the lipophilic chelating agents fusaric acid and quinaldic acid. The genetics of spontaneous antibiotic resistance in E.coli is complex: George and Levy (1983b) identified 3 other loci in addition to the marA locus that were involved in the elaboration of higher levels of resistance.

Tetracycline resistance was found to be due to an energy dependent efflux of tetracycline. This appeared to be specific for tetracycline. Chloramphenicol accumulation was much reduced in marA mutants, but it was not actively pumped out of the cell; and the accumulation of ampicillin was not affected. Their results suggest that marA may normally act as an "environmental locus" controlling the ingress and egress of substances of dubious value to the cell.

The MAR mutants described here differ from those of George and Levy (1983a) in only one respect: their tetracycline resistant mutants were stable over periods in excess of 200 generations of growth in the

absence of selection. After a comparable period, my MAR mutants, with a stability of 97% per cell generation would have shown only 0.2% antibiotic-resistant. This reversion would be very high for a point mutation, so it seems most likely that the MAR mutations described here are due to duplications or unstable insertions.

Another way in which the results of George and Levy (1983a) differ from my results is their observation that when they selected resistance to 5µg/ml tetracycline, the Tet^R mutants invariably also became chloramphenicol resistant. I, however, found mutants that were resistant to tetracycline and ampicillin, but not to chloramphenicol (TAR). One of the factors that may have influenced this is temperature of selection. The TAR mutant was selected at 30°C (although it remained temperature resistant at 37°C). George and Levy (1983a) found that they could not select tetracycline resistance at 42°C, although in that case the tetracycline efflux system was found to be temperature-sensitive, and <u>marA</u> mutants could not be propagated at 42°C on tetracycline.

A last difference from the results of George and Levy (1983a) is the selection of ND15 MAR14. They found that they could not select directly for resistance to 10µg/ml of tetracycline, which was the concentration used to select MAR14. This may however reflect the decay of tetracycline in the selective plates, rather than an actual difference in intrinsic levels of antibiotic resistance, since ND15 grew up after 72 hours' incubation at 37°C. Nonetheless, the levels of resistance to antibiotics shown by ND15 are unlike those seen by George and Levy (1983a). In particular, ampicillin resistance is some 10 fold lower in ND15 than in their mutants, so it seems that MAR14 is a different class of multiple antibiotic resistance mutation.

The isolation of chromosomal mutations in these experiments, rather than the desired ori-plasmids, clearly resulted from an over-cautious use of antibiotic. Later experiments on ND91, a strain with a chromosomally integrated copy of the pBR325 derivative pND5 (see Chapter 3) showed that a single copy of pBR325 could give resistance to at least 50µg/ml

ampicillin and 10μg/ml tetracycline. Use of these antibiotic concentrations would presumably have prevented the growth of, or at least drastically reduced the number of, chromosomal mutants. Nonetheless, it is desirable to use antibiotic concentrations lower than these, since selection of tetracycline resistant transformants with pSC101 (which carries the same tetracycline resistance determinant as pBR325; Bolivar et al.,1978) showed that the yield of Tet^R transformants was two to four-fold reduced when the tetracycline concentration in selective plates was increased from 5 to 10 μg/ml (Table 5.7).

Where, as in these experiments, selection for a rare class of recombinants is being made, it is clear that the use of the higher level of tetracycline would result in a significant decrease in the efficiency of recovery of the desired transformants. The results of George and Levy (1983a, 1983b) suggest two solutions to the problem of optimising yields of transformants while avoiding excessive numbers of chromosomal mutants: One is to work at 42°C where spontaneous Tet^R mutants do not occur; the other is to use a strain carrying the marA::Tn5 insertion, which reduces spontaneous mutation to tetracycline resistance to undetectable levels, but leaves Tn10-based tetracycline resistance and Tn9-based chloramphenicol resistance unaffected.

A number of questions remain outstanding concerning the results reported above:

1) Do the MAR mutants map to the chromosomal marA locus?

2) Are the other tetracycline-resistant mutants dependent on marA function - does introduction of the marA::Tn5 mutation abolish tetracycline resistance?

3) Are these mutants temperature-sensitive for antibiotic resistance?

4) Do the putative lon mutations SAR8 and MAR14 indeed map to the lon locus?

Table 5.7 : Effect of antibiotic concentration on yield of Tet^R transformants.

	tetracycline concentration in selective plates (µg/ml)			
	1	2	5	10
ND13(polA) x pSC101	confluent	+	1167	606
ND13(polA) control	confluent	+	1	0
ND11(polA+) x pSC101	confluent	+	3545	897
ND11(polA+) control	confluent	+	0	0

+ indicates the presence of isolated colonies too numerous to count.

5) Which, if any, of pND4 and its derivatives contain inserts of *Alcaligenes faecalis* DNA? Does tetracycline resistance in ND17 behave as a chromosomal marker in matings and transductions as would be expected if pND4 were integrated into the chromosome?

It may be noted that if the putative *lon* mutations do map to the *lon* locus, then selection for low-level resistance to inhibitors of protein synthesis may provide a means of selection for *E.coli* strains defective in protein degradation. Such mutants may be of some commercial interest, since proteolysis is a major barrier to obtaining high yields of foreign proteins in *E.coli*.

In summary, attempts to isolate origins of DNA replication from *Alcaligenes faecalis*, *Morganella morgani*, and *Vibrio natriegens*, have yielded a wider range of tetracycline-resistant mutants than have previously been reported, but the hoped for ori plasmids proved elusive. Two plasmids which might replicate via an *A.faecalis*-derived origin have been detected, but at present the weight of evidence suggests that these plasmids do not form autonomous replicons in *polA* cells.

Table 5.8 summarises the properties of the various antibiotic-resistant strains.

Table 5.8: Properties of Tet^R strains

strain	presumed antibiotic resistance factor	presumed origin	stability	morphology
ND17	pND4	hybrid between *A.faecalis* DNA and pBR325	80%	heterogeneous
ND21	pND3	"	65%	normal
ND22	MAR5	chromosomal mutation	97%	normal
ND24	MAR7	chromosomal mutation	97%	normal
ND18	SAR8	chromosomal mutation	stable	long cells
ND19	TAR4	chromosomal mutation	97%	normal
ND23	TCR3	chromosomal mutation	unstable	normal
ND15	MAR15	chromosomal mutation	unstable	filamentous
ND160 ND161 ND162 ND163	pND160 pND161 pND162 pND163	chromosomally integrated *oriC* pBR325 hybrid	>99.5%	normal
ND1202	pND02	chromosomally integrated *oriC*-pBR325 hybrid	NT	NT
ND1201	pND01	Hybrid between pBR325 and *E.coli* DNA. Probably not *oriC*.	NT	NT

Chapter 6. Discussion: Suppression of dnaA by λsidA

The data presented in Chapter 3 show that λsidA carries the groE gene. This conclusion is based on the following observations:

(i) sidA maps to a genetic locus roughly equidistant between purA and mel, which corresponds to the genetic locus of groE. (Guest & Nice, 1978)

(ii) The size of the EcoR1 insert in λsidA is the same as that in the *in vitro* constructed λ-transducing phages λgt-Ec-groE (Hendrix & Tsui, 1978) and W3 (Georgopoulos & Hohn, 1978), which have been shown to encode the 65kd groE(L) polypeptide.

(iii) λsidA is able to form plaques on groE- bacteria unable to plate normal λ phage.

Louarn *et al* (pers. comm.) have reported dnaA suppression by a similar phage, and they attribute this specifically to the groEL gene. However, since the data on which they base this conclusion is not currently available, it seems appropriate to consider the possibility that some other gene might be involved, before embarking on a discussion of the possible mechanisms of dnaA suppression by the groE product.

The genetic bounds of the sidA fragment are set by the ampC gene, whose restriction map is known (Edlund & Normark, 1981) and which, on this basis, does not overlap the sidA fragment; and the aspA gene, which is not complemented by λsidA. These are the two closest accurately mapped genes to groE (Bachmann, 1983) so if any other known genes are encoded by λsidA, they will be found among genes whose exact position relative to other nearby genes is not known. Those near enough to be worth considering are: poxA (the regulatory gene for pyruvate oxidase (poxB), Chang & Cronan, 1982); dgkR, the regulatory gene for diglyceride kinase (dgkA) (Raetz *et al*., 1981); pheR, the regulatory gene for pheA (chorismate mutase/prephenate dehydratase

(Gowrishankar & Pittard, 1982), the control step in phenylalanine biosynthesis); bymA, mutations in which allow uptake of maltose in the absence of malT activity (Hofnung & Schwartz, 1971); alr (alanine racemase), which generates the d-alanine required for cell wall biosynthesis (Wijsman, 1977); and leuV, a duplicate gene for leucine tRNA1 (also encoded by leuT (84')) (Ikemura & Ozeki,1977). None of these genes seems a likely candidate for a dnaA-suppressor, although it might be worthwhile screening λsidA for complementation of these genes in the interests of obtaining more accurate map locations for them.

Thus, if dnaA-suppression by sidA is not due to groE, it is probably due to a presently unidentified gene. Only 3 polypeptides are known to be encoded by the 8.2kb groE EcoR1 fragment: gp groEL (65kd), gp groES (15kd) and an unidentified 75kd polypeptide transcribed in the opposite direction to groEL (Hendrix & Tsui, 1978; Tilly et al. 1981); however, the abundance of the groE gene products may have caused minor polypeptides to be overlooked, as was initially the case for gp groES (Hendrix & Tsui, 1978); certainly the 8.2kb fragment has coding capacity for several more polypeptides.

The results of Takeda & Hirota (1982) indicate that the groEL protein is necessary, if not sufficient, for suppression of dnaA, since in mutants of the groE plasmid pYT47 defective for dnaA-suppression, they demonstrated an altered isoelectric point of a protein, whose size they measured as 68kd. Although this is some 3-5kd larger than the size usually reported for the groE protein, the great abundance of this protein in minicells carrying pYT47 makes it unlikely that it could be any other protein.

Nonetheless, it seems entirely plausible that the groES gene is also required for dnaA suppression. The availability of temperature-sensitive mutations in both groEL and groES should render correlation of loss of dnaA-suppression with loss of groEL or groES function in λsidA or pND5 a relatively straightforward matter. Furthermore, derivatives of λgroE specifically deleted for groEL or groES, or carrying mutations in either of these genes have been constructed (Tilly et al.,1981) and it would be

instructive to investigate the ability of these phage to suppress dnaA.

Both groEL and groES are essential for E.coli growth, at least at high temperatures, since temperature-sensitive lethal mutations in both genes have been identified (Georgopoulos & Eisen,1974; Tilly et al.,1981; Wada & Itakawa,1984). At high temperatures, groEL mutants make aseptate filaments (Georgopoulos & Eisen,1974) and mutants in both genes exhibit a decline in the rate of RNA and DNA synthesis, although protein synthesis continues for some time (Wada & Itakawa,1984). The groES and groEL gene products appear to interact in vivo (Tilly & Georgopoulos,1982), although no complex between them has been identified.

The products of both genes are required for the morphogenesis of the bacteriophages λ and T5, but only groEL seems to be necessary for T4 morphogenesis (Tilly & Georgopoulos, 1982). In contrast to their effect on E.coli cell growth, groE mutants do not affect phage macromolecular synthesis (at least, at temperatures permissive for cell growth and restrictive for phage production).

T5 is unable to assemble tails in groE mutants, while λ and T4 are defective in head assembly.

In λ, the block to head assembly is at an early stage, during the assembly of the "preconnector", a precursor of the head-tail connector which appears to nucleate polymerisation of the headshell. In the presence of active groE product, the products of genes B, C, Nu3 and E interact to give a prohead, containing gpB, a proteolysis product, gpB*; 2 fusion-cleavage derivatives of gpC and gpE, and the major coat protein, gpE, into which DNA is subsequently packaged. In the absence of groE, in place of the icosahedral prohead, tubular structures (polyheads) and proheads of excessive size (monsters) are formed, and no processing of gpB to gpB* occurs (Murialdo & Becker, 1978).

In T4, the major coat protein, gp23 accumulates at the membrane in "lumps" in groE cells. This protein is insoluble in the cytoplasm

without the phage gene 31 protein and the groE protein. A similar effect is caused by mutants in the fatA gene (Simon et al.,1975).

In addition to interacting with T4 gp31, λgpB (with which it forms isolatable complexes (Murialdo,1979)), and the groES gene product, the groEL protein also copurifies with glutamine synthetase (Burton & Eisenberg, 1980), RNA polymerase (Ishihama et al., 1976; Paetkau and Coy, 1972) and ribosomes (Subramian et al., 1976). To what extent these results reflect in vivo interactions of groE, and to what extent they merely reflect the fact that a protein as abundant as the groE protein is always likely to be a contaminant in protein preparations, is not yet clear.

The groE genes are heatshock genes, transcribed from a common promoter under the control of the heatshock regulatory gene, htpR (Yamamori & Yura,1982). The heatshock genes are induced by nalidixic acid and UV irradiation, but most conspicuously by sudden temperature increase, where the rate of synthesis transiently increases as much as 50-fold before settling down to a higher level at the new temperature (Neidhardt et al.,1983).

Fig. 6.1 shows a proposed control circuit for the heatshock regulon. htpR, the positive regulator bears striking sequence similarity to rpoD σ-factor, although it is only half the size of the latter protein (Landick et al.,1984), and has been shown to be active as a σ-factor, with specificity for heatshock promoters (Grossman et al.,1984), among them its own promoter; thus htpR is a positive regulator of its own synthesis. The negative element in this circuit is dnaK, whose product is a protein kinase (Zylicz et al.,1984) of an extremely highly conserved class which shows 50% homology to its Drosophila analogue (Bardwell and Craig,1984). dnaK is thought to act by phosphorylating, and thus inactivating, the htpR product (Tilly et al.,1983).

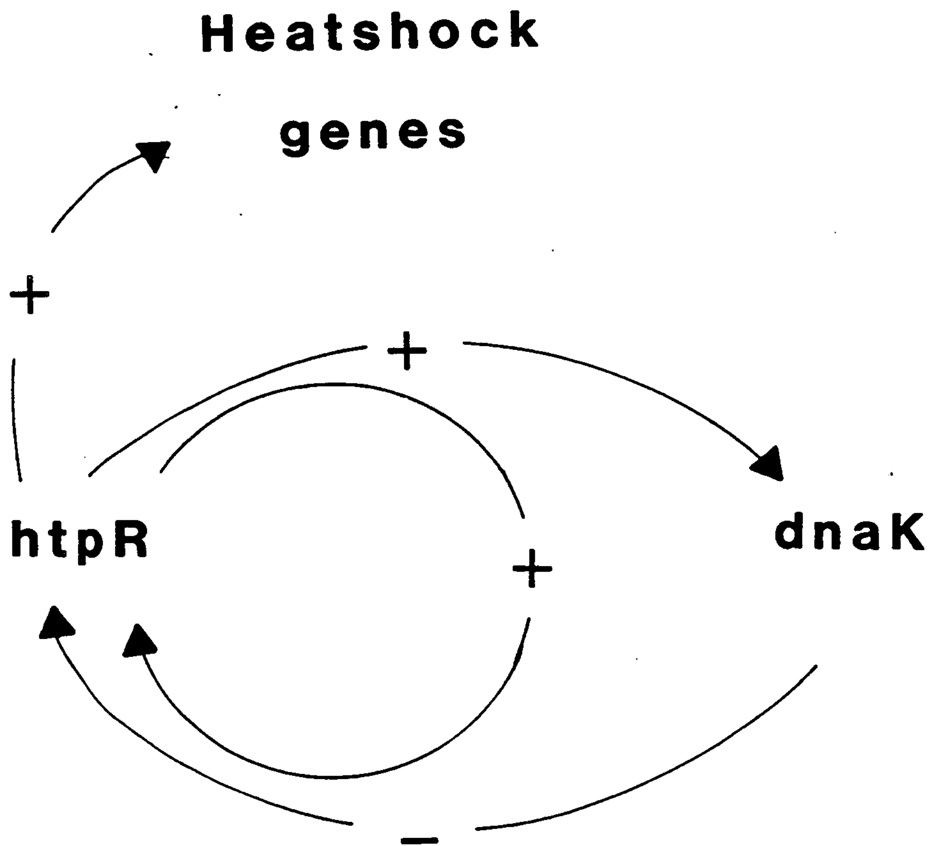
17 heatshock polypeptides are currently recognised (Neidhardt et al., 1984), but not all of these have yet been assigned to genes. Those that have are groE(L), groE(S), dnaK (a protein kinase), htpR and rpoD

Figure 6.1. Proposed control circuit for heatshock regulation

The *htpR* protein (σ32) is an RNA polymerase σ factor specific for heatshock promoters (Taylor *et al.*,1984). A heatshock causes increased activity of σ32 which amplifies by positive feedback due to increased transcription of *htpR* mediated by increased σ32 activity. The increased activity of σ32 also causes increased expression of the heatshock genes. Among these is the *dnaK* gene, a negative modulator of the heatshock response which appears to interact with *htpR* and counteract its stimulatory action on heatshock promoters (Tilly *et al.*,1983). Thus, in *dnaK* mutants, the heatshock response, once turned on, cannot be turned off again.

These elements of both positive and negative feedback allow for rapid and accelerating induction of the heatshock response, but, by induction of the negative regulator as part of the response, also allows for the subsequent switching off of the response.

Figure 6.1



Suggested control circuit for heatshock regulation

(σ-factors), lon (the major E.coli protease) and lysU, a lysyl tRNA synthetase. Why this particular diverse spectrum of proteins is induced may become apparent when the identities of the other 11 proteins are known. Heatshock induction is adaptive, in that cells which have been previously induced for the heatshock response survive better at 55°C (Yamamori & Yura, 1982). Since stressful treatments in general (nalidixic acid, UV (Krueger & Walker, 1984), ethanol, coumermycin induce the heatshock response (Travers & Mace, 1982)), it would appear that heatshock proteins in some way render cells more "robust".

The groE protein is a large acidic protein of approximately 65kd with a weak ATPase activity and is unusual, perhaps unique, among E.coli proteins in forming an oligomeric complex with 7-fold rotational symmetry (Hohn et al., 1979). This complex is composed of 14 monomeric subunits, which are highly asymmetrical cylinders, stacked in 2 rings of seven. Like dnaK, groE appears to be a conserved protein, since similar proteins have been isolated from peas (Pisum sativum, Puskin et al., 1982) and Bacillus subtilis (Carrascosa et al., 1982). Interestingly, however, it is the monomeric form which copurifies with ribosomes (Neidhardt et al.,1981).

Sevenfold symmetry has some interesting consequences: unlike the triangle, the square and the hexagon, the heptagon, having an internal angle at its vertices that is not a factor of 360°, cannot form flat aggregates; nor, since the internal angle is >120°, is it possible for 3 heptagons to adjoin vertex to vertex, so that it cannot, like the pentagons in a dodecahedron, assemble into a close packed 3 dimensional figure. Any larger array of groEL oligomers will thus always contain substantial interstices, and, no matter how great its concentration, it will never crystallise in its multimeric state.

What the significance of this structure is, is not clear, although the fact that the λ head, the first "facet" of which is the preconnector (which contains gpgroEL), has a triangulation number of 21 (3 x 7) may be more than numerical coincidence. In the geometrically more complex T4 head, the value Q, related to triangulation number is

also 21.

The very limited information that is available on the biochemical action of the groE protein and its cellular function means that a wide range of possible explanations for suppression of dnaA by groE is possible, some of them suggested by experiments described in Chapter 3, and some by the brief description of the properties of groE mutants given above.

Surprisingly, although these results implicate the groE genes in dnaA suppression, none of the das suppressors described by Atlung (1981) maps near to groE, although a spontaneous duplication of this region would be expected to suppress dnaA.

Edlund and Normark (1981) have described amplifiable duplications of the neighbouring ampC gene, although those which they characterised did not extend into the groE region. It would be of some interest to know (a) whether spontaneous dnaA-suppressor mutations at the groE locus can be isolated and (b) whether these are invariably duplications or whether it is also possible to isolate groE missense mutants suppressing dnaA, in which case a specific interaction between the two proteins would be implied.

The mode of regulation of the heatshock genes indicates that overexpression of groE could also be achieved by amplification of htpR (as has been demonstrated by Grossman et al.,1984) or mutations in the negative regulator element, dnaK.

I have examined the restriction map of the htpR region (Neidhardt et al.,1983) and compared it with the multicopy suppressors of dnaA shown in fig. 1.3. None of the restriction patterns appears to be congruent with that of htpR; neither do any of the mapped dnaA suppressor mutations map close to this gene. The dasE mutation does map close to dnaK, but Atlung (1981) specifically excluded this locus by genetic mapping.

It would be of considerable interest to see whether plasmids overexpressing the htpR gene can suppress dnaA, as we would expect.

Consideration of the other currently known dnaA-suppressor loci indicates that groE is the only known heatshock locus which corresponds to a dnaA suppressor.

Possible mechanisms of suppression

1. Bypass suppression

The complexity of the interaction between pND5 and the varying genotypes of its hosts means that although some dnaA alleles are not suppressed by sidA, we cannot at present be certain that this is due to allele-specificity of suppression rather than host-specificity. Evidently, it is a matter of some urgency to test for the suppression of null alleles of dnaA such as ambers, or the recently isolated dnaA850::Tn10 allele (Kogoma & von Meyenburg,1983), in a background in which we know suppression can take place.

The possibility that sidA is a dnaA-bypass suppressor is thus still tenable. Mechanisms of bypass suppression have been discussed in the introduction to this thesis. As has been noted, the fact that suppression by sidA plasmids can occur in a recA background (Takeda & Hirota, 1982) eliminates the possibility that integrative suppression is occurring. It might, however, be of interest to test whether inhibition of protein synthesis inhibits DNA synthesis in sidA strains to test whether a form of stable DNA replication is occurring in sidA strains, although this process has also been found to be recA dependent.

2. Control of the dnaA gene

As has been noted, the phenotype of dnaA46 pND5 strains is reminiscent of that of strains overproducing the dnaA46 mutant product; that is, they are cold-sensitive and temperature-resistant (von Meyenburg et al.,1985 in press).

Fusions have been made between the dnaA promoter and the β-galactosidase gene (Atlung et al.,1985 in press). It would be interesting to investigate the effect of sidA on expression of these fusions.

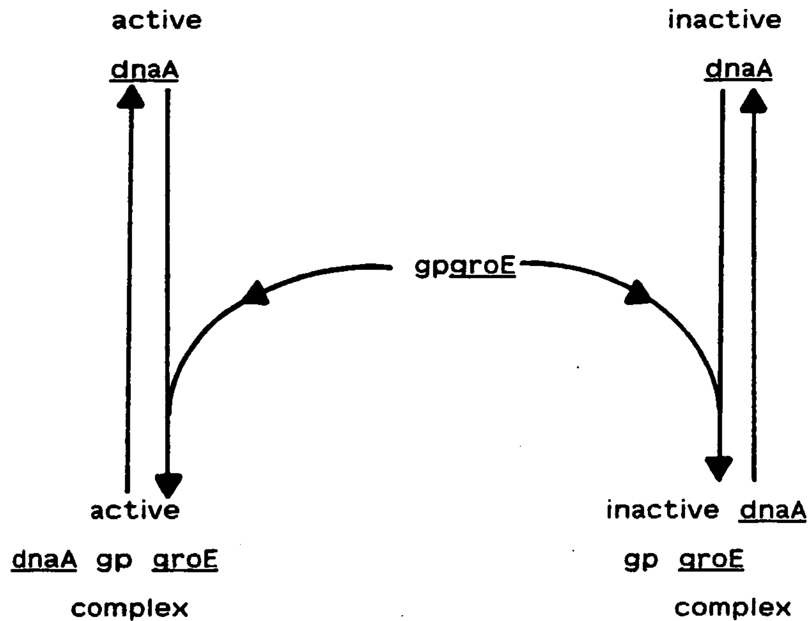
Since the gyrB gene is downstream of the dnaA gene, it may be that the nalidixic acid sensitivity of dnaA strains results from an imbalance of gyrase subunits resulting from enhanced transcription reading through from the dnaA gene. In this context it is interesting to note that dnaA strains, which overtranscribe dnaA due to a failure of the mutant product to give full autorepression (Atlung et al.,1985, in press), also show a reduced resistance to nalidixic acid (Filutowicz,1980). The effect may also, however, be due to the effect of groE on the permeability of the cell mentioned below (6).

3. Modification of RNA polymerase action

At high temperatures in temperature-sensitive groEL and groES mutants RNA synthesis is inhibited. This suggests that dnaA suppression might be mediated via an effect of overproduction of groE on RNA polymerase action. One possible route for such a suppression mechanism would be via expression of the dnaA gene as suggested above. Alternatively, the groE protein might modify the action of RNA polymerase in a manner analogous to the effect of the dnaA-suppressing rpoB mutations, so that, for instance, pausing at the oriC terminator was enhanced. If this is the case, then one might expect that, by analogy with the relevant rpoB mutants (Schauss et al.,1981; Atlung & Hansen,1983), termination at the trp attenuator would be enhanced in strains carrying pND5.

4. Specific interaction with dnaA protein

The possibility of a specific interaction between the dnaA protein and gp groE in initiation and correction of the dnaA(Ts) defect by a "mass action" effect where, according to the scheme set out below, increase in groE concentration increases the concentration of the active dnaA-gpgroE complex, cannot be ruled out.



No evidence for a requirement for the *groE* gene product in the *in vitro* initiation system has been reported, but considering the great abundance of this protein, it seems quite possible that it would be a contaminant of one or more of the purified enzyme preparations used. The temperature sensitivity of DNA synthesis in *groE*(Ts) mutants lends support to the idea of direct involvement of *groE* in DNA synthesis, but the effect could equally well be indirect via inhibition of RNA synthesis. It is unfortunate that the experiments of Wada & Itakawa (1984) used pulse labelling rather than continuous labelling to investigate the effect of inactivation of *groE* on DNA synthesis, since it is not easy to see whether their results reflect an immediate decline in DNA synthesis or the residual DNA synthesis characteristic of initiation mutants.

5. Protein processing

In both λ and T4 grown in *groE* hosts, the processing of virion proteins is defective. In λ, the minor coat protein gp*B* is not cleaved to gp*B** (Murialdo & Becker,1978); while in T4, cleavage of one of the tail fibre proteins is defective (Binkowski & Simon,1983). However, there is no reason to believe that the *groE*-dependent T4 head morphogenesis

requires protein processing.

Nonetheless, the possibility that groE either directly or indirectly catalyses the processing of the dnaA46 gene product to an active cleaved form might be considered. Under such a scheme, the inactive dnaA46 gene product would be regarded as being unusually resistant to processing and therefore to require additional groE protein to drive the reaction. Clearly, if such were the case, then a change in the electrophoretic properties of the mutant protein in SidA compared with normal backgrounds would be expected.

6. Membrane interactions

A number of findings, mostly relating to the cellular location of partially-processed T4 particles, point to an association of gpgroE with processes at the inner membrane. T4 gp23 accumulates in "lumps" at the cell membrane and the "release" of such material into T4 heads by a gp31-gpgroE complex is thought to occur at the cell membrane, especially since mutations in fatA (a membrane protein) exhibit a very similar phenotype (Simon et al.,1975). Furthermore, groE mutants are unusually sensitive to deoxycholate (Georgopoulos et al., 1972) and resistant to certain toxic tripeptides (Takano & Kakefuda, 1972). If gpgroE is a participant in membrane structures then overexpression of this gene could lead to an enhanced persistence or stability of a membrane-associated initiation complex.

7. Physicochemical stabilisation

The great abundance of the groE protein in E.coli cells, which rises to 12% of total cellular protein at 46°C (Herendeen et al., 1979) seems to speak in favour of the protein, if it has enzymic activity, being needed in a particularly active cellular process. This is almost certainly RNA synthesis, since protein synthesis proceeds in its absence at high temperature (Wada & Itakawa, 1984), and DNA synthesis (which is groE dependent) is, in terms of the molar quantities of the factors involved, a very minor process. RNA synthesis can, however, proceed in

vitro in the absence of groE protein, which suggests that the effect of groE on RNA synthesis may be indirect.

An alternative hypothesis, provoked by the great abundance of this protein, its remarkable structure, and its role in virus capsid assembly, is that the role of the groE protein in the cell is a structural one. Three ways in which dnaA suppression might result from such a role are suggested.

1) groE protein may be structurally involved in many subcellular structures, including the initiation complex, so that excessive production of this protein results in more frequent, more rapid, or more efficient assembly of such complexes, and thus a more efficient use of the limited dnaA activity remaining in the cell at high temperature.

2) groE protein could act as a sort of "macromolecular splint", which is able to interact with a large number of proteins and stabilise them, thus lending structural support to them at high temperatures. This would both explain its suppression of dnaA(Ts) and account for its great abundance at high temperatures. Since groE protein is required for RNA synthesis at high temperatures, and copurifies with RNA polymerase, one would expect that temperature-sensitive mutations in RNA polymerase would be suppressed by amplification of sidA.

3) Due to their heptagonal structure, aggregates of groE multimers will always contain voids with dimensions considerably larger than the size of the multimers themselves. It may be that when the concentration of groE protein is very large the division of the cytoplasm into such pockets may alter its physicochemical properties in such a way as to moderate the effects of thermal vibration and thus bring about a general stabilisation of all proteins.

The prediction of all these models is that amplification of sidA

will suppress a wide range of temperature-sensitive mutations. At present, mutations in dnaA, dnaB, dnaC, and dnaP, have been examined, and only dnaA mutations are suppressed. It would be interesting and straightforward to test a large random sample of temperature sensitive mutations for suppression by sidA.

Multiple phenotypic effects of amplification of sidA

Amplification of the sidA fragment results in a number of phenotypic effects besides suppression of dnaA and complementation of groE. So far, reduced growth rate, increased sensitivity to nalidixic acid in NalR strains, and (under some circumstances) a disturbance of cell morphology have been observed.

Consideration of the possible consequences of amplification of the groE gene to pBR325 copy numbers shows that these results are hardly surprising. If one naively assumes that the quantity of gpgroE in the cell is directly proportional to copy number, and moderately estimates the copy number of pND5 to be 20, then the lowest estimate of cellular gpgroE concentration described by Neidhardt et al. (1984) of 0.8% total cellular protein would become 16% due to gene amplification. Although this is probably a gross overestimate, since it fails to take into account such factors as possible limiting concentrations of htpR protein, it is clear that we may expect plasmids carrying groE to cause significant alterations in the cellular protein composition. The results of gel electrophoresis of total cellular protein in strains amplified for sidA, to estimate the degree of amplification of groE protein, would thus be of some interest.

It is not, at present, known whether all the observed phenotypic effects of amplification of sidA are caused by a single gene. Since, however, two of the phenotypic effects (slow growth rate and naladixic acid sensitivity) confer a selective disadvantage on the host cell, it should be possible to select mutations in pND5 which no longer cause these effects, and therefore answer the question of whether dnaA-suppression, slow growth rate, nalidixic acid sensitivity, and

complementation of groEL or groES, are concomitantly lost.

Awareness of these phenotypic effects was essentially a consequence of chance observations made during manipulation of sidA strains. It therefore seems likely that examination of any of the more easily measured phenotypic characteristics of sidA strains will reveal other effects. In view of the known phenotypic effects of groE mutations, sensitivity to deoxycholate, toxic tripeptides, bacteriophage, and inhibitors of RNA and DNA synthesis, are obvious candidates for examination. Such a study would have the dual value of defining the "sphere of influence" of sidA and of helping to eliminate from otherwise straightforward experiments the consequences of unforeseen effects of sidA overexpression, which proves to be a continuing problem in work with this gene.

The effects of host genotype on the consequences of sidA amplification also merit attention. In this respect there is a need to differentiate between the consequences of dnaA suppression, the effects of different degrees of dnaA suppression, and interactions between sidA and the host which have no bearing on dnaA suppression. The effect of the zjb501::Tn10 insertion which seems to reduce either suppression efficiency or host tolerance to λsidA, and which maps very close to dnaA, merits further attention, as does the question of how host adaptation, the restoration of near-normal growth rates in initially slow growing transformants carrying pND5, occurs.

In summary, it seems that the effects of overexpression of groE may be very complex, and although certain simple hypotheses about the mechanism of dnaA suppression should be tested, it seems as likely that the effect of groE on dnaA is indirect;, so that the most fruitful approach would be to catalogue the numerous phenotypic consequences of groE overexpression and hope thereby to chart a path through a web of interactions that leads back to dnaA. With luck that path will be a short one; it will surely not be a dull one.

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