The Leading Region of IncI1 Plasmid ColIb-P9

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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Abstract

The leading region of IncI1 plasmid ColIb-P9.
Anna Louise Jones.

The IncI1 conjugative plasmid ColIb-P9 carries a *psiB* gene that prevents induction of the SOS response in host bacteria. This locus was found to be located 2.5 kb downstream of the *ssb* (single-stranded DNA-binding protein) gene in the leading region of ColIb. This portion of the plasmid is transferred first to the recipient cell during conjugation and is strikingly similar to part of the leading region of the otherwise distinct F plasmid. Determination of the nucleotide sequence of ColIb *psiB* demonstrated that the gene has 84% identity with the *psiB* gene of F. Promoterless *lacZ* fusions on ColIb were created to leading region genes *ssb* and *psiB* and to *sog*, a representative transfer gene. It was found that expression of all three genes is increased when the ColIb transfer system is derepressed, but *ssb* and *psiB* are expressed at a much lower level than *sog*. Expression of *psiB* and *ssb* is increased when the host cell is exposed to UV-irradiation or mitomycin C treatments. The DNA-damage inducibility of *ssb* and *psiB* is *recA* and *lexA*-independent showing that neither gene is a component of the SOS regulon. Expression of both *psiB* and *ssb* is strongly enhanced in conjugatively infected recipient cells. No enhanced synthesis of Sog polypeptides was detected following conjugation showing that the zygotic induction of *ssb* and *psiB* is not a general property of plasmid genes. The implication is that PsiB and SSB proteins function in the transconjugant cell, rather than in the primary donor. It has been proposed that PsiB acts to prevent triggering of the SOS response during conjugation by transferring single-stranded DNA. Consistent with this hypothesis, carriage of the *psiB* gene by ColIb was shown to prevent a low level of SOS induction following conjugation. Plasmids that carry *ssb* and *psiB* genes have replicons belonging to the RepFIC family. It is postulated that the trigger for SOS induction during conjugation may be generated during the initial replication of the plasmid in the newly infected recipient cell rather than by the process of single-stranded DNA transfer.

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Abbreviations

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Cm^r	Chloramphenicol resistance
DNA	Deoxyribonucleic acid
dATP	Deoxyribo-adenosine triphosphate
dCTP	Deoxyribo-citosine triphosphate
dGTP	Deoxyribo-guanidine triphosphate
dTTP	Deoxyribo-thimidine triphosphate
drd	Derepressed for transfer
EDTA	Diaminoethanetetra-acetic acid
Inc	Incompatibility group
IPTG	Isopropylthio-β-D-galactoside
kb	Kilobase
kD	Kilodalton
Km^r	Kanamycin resistance
Nal^r	Nalidixic acid resistance
ONPG	Ortho-nitryl-phenyl-galactoside
PEG	Polyethylene glycol
RNA	Ribose nucleic acid
SDS	Sodium dodecyl sulphate
SGC	Salts-glucose-casamino acids medium
SSB	Single-stranded DNA-binding protein
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate-EDTA
Tc^r	Tetracycline resistance
TEMED	N,N,N',N'-tetramethylethyleneediamide
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Chapter 1.
General Introduction.

1.1. Preface.

Bacterial conjugation is a plasmid-encoded mechanism that supports the transfer of a specific plasmid strand from a donor cell to a recipient cell which are in contact. The process is replicative and involves synthesis of a replacement strand in the donor and complementary strand in the recipient to regenerate a double stranded plasmid (Willetts and Wilkins, 1984). The subject of this work is ColIb-P9, which is a large (93 kb) enterobacterial conjugative plasmid of the I1 incompatibility group (Rees *et al.*, 1987). The DNA segment entering the recipient cell first in conjugation is termed the leading region. It is the function and regulation of expression of two leading region genes on ColIb that is under investigation. These genes are *psiB*, determining a protein that inhibits the bacterial SOS response, and *ssb*, specifying a single-stranded DNA-binding protein of the type that binds to DNA without sequence specificity. Homologous *psiB* and *ssb* genes are found on approximately half of the conjugative plasmid groups determined by incompatibility (Golub *et al.*, 1988). The genes products have been implicated to have a role in conjugative DNA metabolism. It has been suggested that plasmid SSBs participate in conjugative DNA synthesis and that PsiB acts to allow ssDNA transfer without triggering the SOS response (Bagdasarian *et al.*, 1986; Howland *et al.*, 1989). Thus, the process of bacterial conjugation and the SOS response of *E. coli* are central to this work and shall be described in detail in this introduction. Of particular importance is the generation of single-stranded DNA during the conjugative process, the subsequent regeneration of double-stranded DNA and the nature of the SOS inducing signal.

1.2. Bacterial conjugation

1.2.1. Introduction

Gram-negative conjugation is a specialised replicative process that increases the population size of a plasmid by its horizontal transfer between organisms. The first conjugative plasmid to be identified was the F fertility factor of *Escherichia coli* (Cavalli-Sforza *et al.*, 1953; Hayes, 1953) and is consequently the best characterised (Ippen-Ihler and Minkley, 1986; Willetts and Skurray, 1987). Since the pioneering discovery of plasmid F, many conjugative plasmids belonging to over 20 incompatibility groups have been identified. However, the conjugation system specified by the F plasmid remains archetypal and its general features are believed to be universal for plasmids that transfer between enterobacteria.

1.2.2. Conjugation as typified by F

The genes required for transfer of F (*tra* genes) are contained within a 33.3 kb Tra region (Fig. 1.1). At one end of this region is the origin of transfer (*oriT*) and at the other, the *finO* regulatory region. The whole sequence of the F Tra region has been determined and a map of the well characterized *tra* genes produced (Ippen-Ihler and Minkley, 1986; Traxler and Minkley, 1987). There are five major transcriptional units within the Tra region (Manning *et al.*, 1984) and most genes lie within the *traY-Z* unit, with *traM*, *traJ*, and *finP* located outside (Willetts and Skurray, 1987).

Conjugative DNA transfer consists of two major processes. The first involves cell surface interactions to bring mating cells into close contact. This process requires conjugative pili to be synthesized by the donor cell. F plasmids specify a long flexible type of pilus. The pilus is thought not to be the route through which the DNA transfer occurs, as addition of SDS after

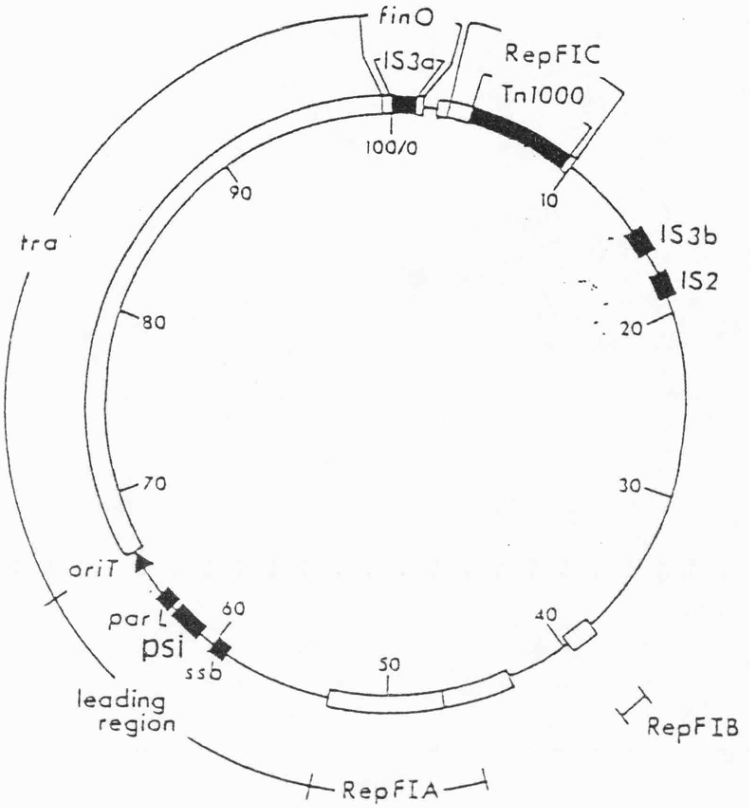
Fig. 1.1. Plasmid F.

(a) Genetic and physical map of the F plasmid. Loci shown are the origin of transfer (*oriT*), *parL* (also referred to as *flm*, F leading maintenance), single stranded DNA binding protein (*ssb*), plasmid SOS inhibition (*psi*), the RepFIA, RepFIB and RepFIC replicons, IS2, IS3b and IS3a insertion sequences and the *finO* regulatory element. The arrow at the origin of transfer, *oriT*, indicates the direction of F plasmid conjugative transfer, with the leading region entering the recipient first and the *tra* region last. (From Willetts and Skurray, 1987).

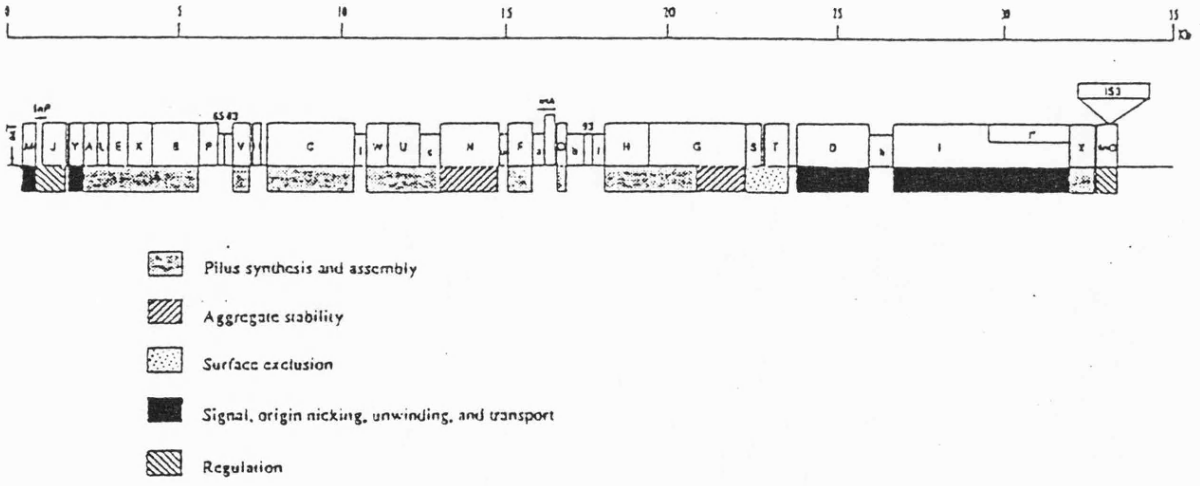
(b) Genetic map of the F transfer region. The map indicates the position of the *tra* genes and open reading frames encoded by the region. (K. Ippen-Ihler, personal communication).

Fig.1.1

(a)


finO
IS3a?
RepFIC
Tn1000
100/0
10
IS3b
IS2
20
30
40
50
60
70
80
90
tra
oriT
par
psi
ssb
leading region
RepFIA
RepFIB

(b)


Pilus synthesis and assembly
Aggregate stability
Surface exclusion
Signal, origin nicking, unwinding, and transport
Regulation

mating pair formation does not affect DNA transfer. SDS has the effect of destabilising the pilin subunits that comprise the pilus. It is generally agreed that after the pilus makes contact with the recipient cell surface, it retracts and brings the cells into closer contact (Willetts and Skurray, 1987). However, two experiments indicate that direct cell-to-cell contact may not be necessary for F-pilus mediated DNA transfer in *E. coli* and that DNA transfer may occur through an extended pilus (Harrington and Rogerson, 1990; Ou and Anderson, 1970).

The mechanism of creating the cell-to-cell contact, although fundamental to the process of conjugation, is of less importance to this work. The second stage of conjugation, that of processing the DNA, and the regulation of *tra* gene expression will be considered in greater detail.

1.2.3. Nicking at the origin of transfer.

Initiation of DNA transfer occurs at a site known as the origin of transfer (*oriT*) and it is at this site that a single-stranded nick occurs. This has been demonstrated *in vivo* by the nicking of λ *oriT* transducing phage in F *tra*$^+$ cells (Everett and Willetts, 1980). These experiments used denaturing agarose gel electrophoresis to separate the λ *oriT* phage DNA strands following infection of F$^+$ cells. One of the λ DNA strands is nicked and therefore two bands corresponding to smaller single-stranded DNA species were observed. The transferred strand of F has been identified as that with the greatest density in poly (U,G)/CsCl gradients (Vapnek and Rupp, 1970) and that this is the sense strand from which the *tra* genes are expressed.

General features of *oriT* sites often include a high AT content and inverted and direct sequence repeats which may act as targets for protein-DNA recognition. The sequence of the F *oriT* has been determined (Thompson *et al.*, 1984) and appears to have different domains contributing

to nicking, transfer and overall structure (Fu *et al.*, 1991). Binding sites for the integration host factor (IHF) of *E. coli* have been detected in the vicinity of F *oriT*. It is proposed that IHF binding may enhance the intrinsic bending of the *oriT* site and aid the formation of higher order structures (Tsai *et al.*, 1990).

Studies on *oriT* nick sites have concentrated on relaxosomes, which are protein-DNA relaxation complexes isolated during plasmid isolation procedures. Treatment with a detergent, such as SDS, releases an open circular DNA species and such procedures have been used to determine the precise nick sites on conjugative plasmids, including that of F (Thompson *et al.*, 1989). There is evidence to suggest that during nicking a specific phosphodiester bond is cleaved between chemically different nucleotides and that the 3′ terminus carries an unmodified hydroxyl group. At the 5′ terminus of the nick site, the strand to be transferred is covalently linked to a plasmid-encoded protein at its 5′ terminal nucleotide. Such linkage consists of a phosphoester with the side chain hydroxyl of a seryl, threonyl, or tyrosyl residue.

Genetic studies of the nicking process of the F plasmid indicated a requirement for the products of *traY* and *traI* (Everett and Willetts, 1980). F *traI* specifies two polypeptides of 180 kD (TraI) and 94 kD (TraI*). TraI* is generated from an internal translational restart and is probably the product of what was previously referred to as *traZ* (Bradshaw *et al.*, 1990; Traxler and Minkley, 1987). The role of TraI* is as yet undetermined. Purified F TraY protein has been shown to bind to double-stranded *oriT* sites in a plasmid specific manner (Lahue and Matson, 1990). F TraI is a multifunctional protein also referred to as DNA helicase I and it has been reported that *in vitro*, purified helicase I alone can mediate specific nicking at F *oriT* in a process that requires superhelical DNA and Mg^{2+} ions. The phosphodiester bond interrupted *in vitro* is identical to that nicked *in vivo*. Additionally

the 3′ terminus of the nick contains a free hydroxyl, but the 5′ is blocked presumably by covalent linkage to helicase I, which also agrees with the *in vivo* data (Matson and Morton, 1991).

The actual processes of conjugative DNA synthesis and DNA transport are initiated in response to a signal generated by the formation of a 'functional' mating pair although the precise nature of this signal is unclear. It is possible that the process of nicking can occur in the absence of recipients and that an equilibrium normally exists between non-covalent complexed supercoiled plasmid DNA and nicked plasmid DNA (Everett and Willetts, 1980). There are other *tra* products that are known to interact with *oriT* sequences but are inessential for the cleavage reaction. F TraM, which binds to three regions in the vicinity of F *oriT*, has been proposed to process the signal that a competent mating pair has formed and subsequently initiate the transfer process. The *traM* gene lies adjacent to F *oriT* and is essential for the transfer process (Achtman *et al.*, 1972; Di Laurenzio *et al.*, 1991).

1.2.4. Single-stranded DNA transfer.

Following single-stranded nicking at *oriT*, the nicked strand is transferred from donor to recipient. In the case of the F plasmid, the transferred strand has been shown to be specific, with a leading 5′ terminus (Ihler and Rupp, 1969; Ohki and Tomizawa, 1968; Rupp and Ihler, 1968). Unwinding of the two plasmid DNA strands, which is a prerequisite for ssDNA transfer, is probably the function of the F TraI protein, which is also known as DNA helicase I (Abdel-Monem *et al.*, 1983). The ssDNA-dependent ATPase activity of the TraI helicase may also act to displace the nicked strand into the recipient cell as the enzyme proceeds unidirectionally 5′ to 3′ relative to the strand on which it is bound (Lahue and Matson, 1988). Thus TraI may migrate on the strand destined for transfer since this has 5′ to

3′ polarity. If TraI was anchored to the membrane, the unwinding reaction could provide the motive force for DNA transport.

1.3. Conjugative DNA synthesis:

1.3.1. Replacement strand synthesis

The conjugative process requires synthesis of a replacement strand in the donor and complementary strand in the recipient to regenerate double stranded plasmids (Willetts and Wilkins, 1984). Kingsman and Willetts (1978) showed that donor conjugative DNA synthesis (DCDS) during F transfer is performed by the *dnaE* product, DNA polymerase III. In principal, DCDS may be primed by the 3′ hydroxyl terminus of the transferring strand. This would extend the strand by a classic rolling circle mechanism (Gilbert and Dressler, 1968). However, there is evidence to suggest that *de novo* synthesis of short RNA primers is necessary for the activity of Pol III in DCDS (Kingsman and Willetts, 1978; Maturin and Curtiss, 1981). The retained strand is unlikely to accumulate in single-stranded form because the rate of DNA synthesis by DNA pol III is ~700 nucleotides per second which is similar to the rate of DNA transport. Interestingly, there is no obligatory coupling between DCDS and the transfer process as *E. coli dnaE* (Ts) mutants are proficient donors of F DNA but are defective in the replacement strand synthesis (Kingsman and Willetts, 1978).

1.3.2. Complementary strand synthesis

DNA synthesis on the transferred plasmid strand is termed recipient conjugative DNA synthesis (RCDS). The process does not require expression of plasmid *tra* genes in the newly infected recipient cell (Boulnois and Wilkins, 1978). The conjugative properties of *E. coli dnaE*

recipients indicates a requirement for DNA polymerase of the recipient cell for DNA synthesis on the transferred plasmid strand (Willetts and Wilkins, 1984). This process utilises *de novo* synthesis of multiple primers as the strand is transferred in the 5′ to 3′ direction. The use of multiple primers should ensure that ssDNA does not accumulate. The effect of rifampicin and *dnaB* inactivation on RCDS of F has shown that the primers are generated by RNA-polymerase or a *dnaB*-dependent process acting as an alternative mechanism. In some conjugation systems, such as those specified by plasmids of the IncI1 and IncP groups, specific plasmid DNA primases are transported from donor to recipient to facilitate priming of RCDS (discussed in detail in section 1.6.1). F does not specify such an enzyme (Rees and Wilkins, 1990).

1.4. Circularization of the transferred DNA.

Circularization of the transferring plasmid is RecA independent (Clark, 1967) and does not require the expression of plasmid genes in the recipient cell. Circularization may involve a mechanism in which the 5′ terminus of the transferred strand remains linked to a membrane protein. This complex may then be able to recognise the 3′ terminus and recircularise the plasmid by ligation (Willetts and Wilkins, 1984). This ligation event may be the reverse of *oriT* nicking and mediated by the Tra protein linked to the 5′ end of the transferring strand (Everett and Willetts, 1980). It is not clear whether proteins at the 5′ terminus are transmitted into the recipient cell or are retained in the donor in the vicinity of the conjugative bridge. The second terminus used for ligation may be the 3′OH group at the trailing end of the transferring DNA. Alternatively, if this terminus created by the original nick at *oriT* is extended by a rolling circle mode of DNA synthesis, then a second nick at the reconstituted *oriT* sequence would be required to generate a unit length of DNA for circularization. Genetic experiments on

the circularization process used plasmids or ssDNA phage M13-based molecules carrying two copies of an IncQ *oriT* as direct repeats. *oriT* recombination in these systems implies there is a mechanism that liberates monomeric single-stranded circles from ssDNA substrates of greater than unit length. The implication is that the 3′ hydroxyl terminus of the nick site is extended to regenerate a complete *oriT* sequence at the trailing end of the transferring strand. Potential secondary structure formed by the inverted repeat in this trailing region may allow recognition of the reconstituted *oriT* (Barlett *et al.*, 1990; Brasch and Meyer, 1987; Kim and Meyer, 1989; Meyer, 1989). However there is no unambiguous evidence that circularization involves processing of a transferred strand that is detectably greater than unit length (Willetts and Wilkins, 1984)

1.5. Regulation of F transfer.

In contrast to the classic F sex factor, most F-like plasmids are repressed for transfer due to the action of the FinOP fertility inhibition system (Meynell *et al.*, 1968). Two structural genes are required for this system to function and while the *finO* product is relatively nonspecific, several alleles of *finP* have been identified. The F plasmid is *finO*/*finP*$^+$ due to the presence of an insertion element (IS3) within *finO* and thus expresses its transfer genes constitutively (Cheah and Skurray, 1986). However, the F *finP*$^+$ product can interact with the *finO* product of a coresident *finO*$^+$ plasmid to produce the FinOP inhibitor (Finnegan and Willetts, 1973). This inhibitor acts to reduce translation of the *traJ* transcript by binding to the *traJ* mRNA. TraJ is the positive regulator required for most *tra* gene expression. Mutants of *traJ* are pleiotropic, being defective in transfer, pilus formation and surface exclusion and in fact the *traJ* product controls expression of the major *traY-Z* transcript of 33 kb (Achtman *et al.*, 1972; Finnegan and Willetts, 1973; Willetts and Achtman, 1972).

FinP is a 72 nucleotide RNA molecule which is complementary to the untranslated leader sequence of *traJ*. These complementary molecules interact together with FinO to inhibit *traJ* expression (Finlay *et al.*, 1986; Mullineaux and Willetts, 1985). The FinP RNA is predicted to have a two stem loop structure and it has been shown by mutation that both loops are involved in the interaction between FinP RNA and *traJ* mRNA (G. Hogenauer, personal communication). These observations may explain the plasmid specificity of *finP*. The *finO* product has been proposed to be a 22 kD protein, which augments the effects of FinP and clearly has an important role as the inhibitory action is only observed in the presence of the *finO* product.

F-like plasmids that are Fin^+, such as R100, normally express only a small fraction of their full potential for transfer and pilus production in established strains but show full expression in newly infected recipient cells (Datta *et al.*, 1966; Meynell and Datta, 1965; 1966; Watanabe, 1963). It was proposed that there was a delay in establishment of the fertility inhibition system controlling *tra* gene expression. Finnegan and Willetts (1971) showed that after transfer of F*lac* ($finP^+$ $finO^-$) to cells carrying the fin^+ F-like plasmid R100, retrotransfer from the primary recipient took place at a high frequency even though these recipient cells already contained a maximal level of the fin^+ products. This therefore suggested that the slow establishment of fertility inhibition may be due to a delay in synthesis of an inhibitory concentration of the plasmid specific *finP* product. However, Willetts (1974) demonstrated that although there is a delay of approximately six hours before inhibition of transfer is established, early synthesis of the transfer inhibitor could be detected. There appeared to be a transient synthesis of the *traJ* product which functions to promote *tra* gene expression even in the presence of *finOP*. Thus transfer inhibition is only established after dilution out during cell growth of the *traJ* product and then the other

transfer gene products. These data are in agreement with the observation that the *finOP* products act to prevent synthesis of TraJ rather than to prevent this positive regulator from functioning.

Since most naturally occurring plasmids are repressed for transfer functions, this system of transitory derepression allows a plasmid to spread rapidly through a population of cells before fertility inhibition is established. (Lundquist and Levin, 1986; Simonsen, 1990). Constitutive expression of the *tra* genes would burden the host cell with an unnecessary metabolic load and also make the cell vulnerable to attack by pilus-infecting phage.

1.6. The I1 conjugation system.

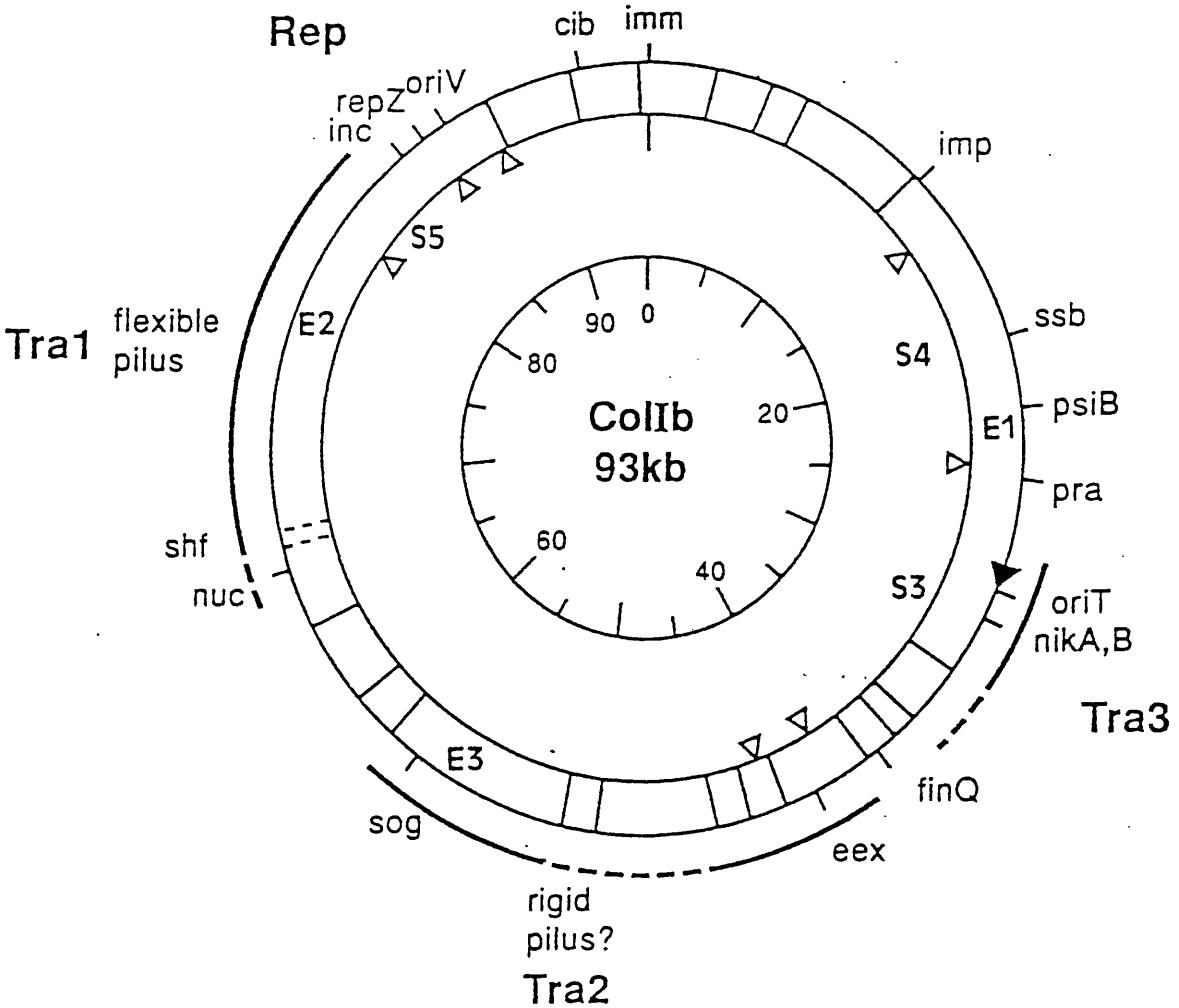
ColIb-P9 is a representative member of the I1 (Iα) incompatibility group of which other members include R64 and R144. These plasmids are thought to specify the same conjugation system, which although functionally similar to that of F and the F-like plasmids, is quite distinct.

IncI1 plasmids and members of the IncB, IncK and IncI2 groups constitute the I complex of plasmids. They differ from F and all other conjugative plasmids studied thus far in specifying two distinct types of sex pilus (Bradley, 1983; 1984). The two types of I1 pilus are a thick rigid pilus thought to be essential for conjugation and a thin flexible pilus, which is immunologicaly distinct from the thick pilus and may function to stabilise mating aggregates in liquid. Both types of pilus are distinct from the F pilus. The I1 conjugation system also includes a DNA primase (Sog), which is involved in processing the transferred plasmid DNA and an EDTA-resistant nuclease of unknown function.

However, in overall function the I1 conjugation system does resemble that of F by involving cell-to-cell interactions mediated by conjugative pili, transfer of a unique single strand of plasmid DNA initiated

Fig. 1.2. Plasmid ColIb.

Loci in the Tra regions determine shufflon (*shf*), and EDTA-resistant nuclease (*nuc*), DNA primase (*sog*), entry exclusion (*eex*), fertility inhibition of the F transfer system (*finQ*) and relaxosome proteins (*nikA, B*). Other loci shown include colicin Ib (*cib*), colicin Ib immunity (*imm*), I group mutation and protection (*imp*). The origin of vegetative replication (*oriV*) and the incompatibility/replication functions (*inc/rep*) are located adjacent to one end of Tra1. Lines within the ring indicate *Eco*RI cleavage sites and triangles show *Sal*I sites. *Eco*RI and *Sal*I restriction fragments of ColIb will be referred to in the text by the letters E and S respectively, followed by the appropriate fragment number, with 1 being the largest. The E1, E2, E3, S4 and S5 fragments, which are relevant to this work, are indicated. The map incorporates data from the study of R64, which specifies a very similar transfer system to ColIb.



at the *oriT* site and DNA synthesis to regenerate double-stranded plasmids in both donor and recipient cells (Willetts and Wilkins, 1984).

1.6.1. Plasmid ColIb-P9.

ColIb-P9 is a large conjugative plasmid (Fig. 1.2) that is characteristically identified by conferring on its host the ability to produce colicin Ib, an antibiotic-like protein (Varley and Boulnois, 1984). Addition of purified colicin to whole *E. coli* cells or membrane vesicles has been shown to induce the formation of ion-permeable channels and the protein causes cell death by depolarisation of the membrane (Weaver *et al.*, 1981a). Cells that express colicin Ib are immune to its effects. The immunity is mediated by a plasmid-encoded inner membrane protein, which is thought to interact stoichiometrically with the colicin after uptake to inactivate it (Weaver *et al.*, 1981b). The gene specifying colicin Ib is known as *cib* (Boulnois, 1981) and its associated immunity gene as *imm* (Gottlieb and Duckworth, 1983). The colicin gene is normally repressed but expression can be induced by DNA damage as part of the SOS response (Glazebrook *et al.*, 1983). Varley and Boulnois (1984) identified a putative SOS regulatory sequence upstream of the 5′ region of *cib*. ColIb has also been shown to confer resistance to the lethal effects of UV irradiation on host enterobacteria (Howarth, 1965) and to suppress chromosomal *umuC* mutations in *E. coli* cells (UV protection and mutagenesis). The locus responsible for these phenotypes is known as *imp* (I group mutation and protection) and is described in greater detail in section 1.7.5.

When bacteriophage T5 infects host cells harbouring a ColIb plasmid, the infection aborts (Strobel and Nomura, 1966). This inhibition requires two genes from the host chromosome (Hull and Moody, 1976), one from the phage (Mizobuchi and McCorquodale, 1974) and one from the plasmid (Pinkerton *et al.*, 1981; Uemura and Mizobuchi, 1982). The combination of

these four gene products cause the cell membrane to depolarize and the infection, although begun normally, to abort (Glenn and Duckworth, 1980). The ColIb plasmid locus is termed *abi* and is located separately from the colicin gene (Pinkerton *et al.*, 1981). The nucleotide sequence of the *abi* locus reveals four possible LexA-binding sites within or downstream of promoters detected by DNA footprinting suggesting that *abi*, like *cib* is a member of the SOS regulon (Gupta and McCorquodale, 1988).

The ColIb *tra* determinants are located in a 50 kb segment (ColIb 29.0 to 79.0; Fig. 1.2). There are three blocks of *tra* genes, although it is possible that Tra2 and Tra3 are functionally one region (Rees *et al.*, 1987). The genes necessary for synthesis of the thin pili are thought to be located in Tra1, in the S1/E2 regions, as transposon insertions generated here abolish thin pilus synthesis (Rees *et al.*, 1987). The efficiency of transfer of such insertion mutants on surface matings is near normal but highly defective in liquid cultures, indicating that the only Tra function affected is the thin pilus. Within the Tra1 region is the highly mobile DNA segment known as the shufflon (Komano *et al.*, 1990). The shufflon apparently acts as a biological switch that modifies the structure of the thin pilus by altering the 3′ coding region. The nuclease gene is located at the end of Tra1 and has no known function but it may be similar to the nuclease gene of IncN plasmids (Lackey *et al.*, 1977; Winans and Walker, 1983). The IncN nuclease gene is not coordinately expressed with the transfer genes and is not essential for conjugation. The Tra1 region of R64 (IncI1) has been sequenced and data comparisons have shown no similarities between this region and the F Tra region which has been sequenced in total (T. Komano, personal communication).

The Tra2/3 region encodes the *sog* gene whose product is the Sog DNA primase. Plasmid primases were originally recognized by their ability to synthesize functional primers on single-stranded DNA from small

bacteriophages and to substitute for bacterial primase during replication in *dnaG* (Ts) mutants of *E. coli* (Lanka *et al.*, 1979; Wilkins, 1975; Wilkins *et al.*, 1981). The best characterised plasmid primases remain Sog of ColIb and TraC (Pri) of the IncP plasmid RP4. As this thesis concerns the ColIb plasmid, I shall concentrate on the Sog primase for the purpose of this introduction. The *sog* gene was chosen as a representative ColIb *tra* gene for regulatory studies described in Chapters 4 and 5. The *sog* gene determines two polypeptides of size 210 kD and 160 kD, as separate in frame translational products differing at the amino-terminus. The primase moiety is located in the unique N-terminal region of the larger polypeptide (Merryweather *et al.*, 1986; Wilkins *et al.*, 1981). Recent sequence studies of the plasmid primases has indicated a motif of EGYATA in the primase domain of RP4 TraC and ColIb Sog. Mutations in this region can either increase, decrease or eliminate the primase enzymatic activity (Bettina Strack, personal communication). However, primase negative mutants do not show strong defects in conjugation (Chatfield *et al.*, 1982).

Both Sog polypeptides are transferred into the recipient cell with approximately 250 molecules of each per transferred plasmid strand (Merryweather *et al.*, 1986; Rees and Wilkins, 1989). It is thought that there is a second function of Sog polypeptides and that is to promote the DNA transfer process. The C-terminal region of the larger Sog polypeptides is identical to the smaller protein and has been implicated in DNA transport. Genetic evidence implies that this second function of the Sog polypeptides is more important in conjugation than the primase activity. Rees and Wilkins (1990) showed that the larger RP4 Pri protein is also transferred to the recipient cell by a mechanism that is presumably similar to that of Sog. However, no such protein transfer has been observed for F-like plasmids (Rees and Wilkins, 1990).

At the end of the Tra2/3 region is the *oriT* site. The *oriT* region of the closely related IncI1 plasmid, R64, has been studied in detail (Furuya *et al.*, 1991). Formation of the R64 relaxosome requires *oriT* and the adjacent trans-acting *nikA* and *nikB* genes. These *nik* genes are arranged in an operon having the same orientation relative to *oriT* as the IncP plasmid relaxase operon (*traJ/traI*). NikA protein shares 30% sequence similarity with TraJ of RP4 and only the N-terminal portion of NikB is required for relaxation, as is the case for RP4 TraI. The R64 *oriT* contains a consensus sequence present at the nick sites of IncP plasmids and in the border sequence of Ti/Ri plasmids (Pansegrau and Lanka, 1991). Clearly the IncI1 Tra2/3 region shows similarities to an IncP type conjugation system and these are particularly evident in the *oriT* region.

1.6.2. Regulation of ColIb *tra* gene expression.

The ColIb *tra* genes are normally repressed, suggesting the possibility of a fertility inhibition system analogous to the FinOP system of the F-like plasmids (section 1.5.). Evidence suggests that repression is mediated by a *trans*-acting factor, since the presence of the ColIb wild type plasmid in a cell containing a cosmid carrying most of the *tra* genes will prevent thin pilus synthesis directed by the cosmid (Rees *et al.*, 1987).

Studies on ColIb have been facilitated by the isolation of derepressed (*drd*) mutants, which presumably have a mutation that affects the fertility inhibition system (Meynell and Datta, 1967). However the exact nature of the *drd* mutations used for the work described in this thesis are as yet undefined. There is evidence that expression of the ColIb *tra* genes requires the action of two positive regulators. Transposon insertions generated in S5 or E18 (located in Tra1 and Tra2, respectively; Fig. 1.2.) show pleiotropic effects, abolishing pilus synthesis, conjugative efficiency and primase gene expression. These are the testable Tra phenotypes. A simplistic model of

ColIb *tra* regulation is that a negative regulator controls synthesis of these two transacting positive regulators, which are required for *tra* gene expression. The *drd* mutation may abolish or reduce the synthesis of the negative regulator, allowing expression of the positive regulators and hence the *tra* genes.

One of the most interesting features of the ColIb conjugation system is its apparent duality. There are two separate pilus assembly systems and *tra* gene expression requires the action of two positive regulators. The *tra* genes are arranged into two, or possibly three separate regions. These observations raise the possibility that the I1 system may have arisen from an ancestral fusion between two ancient conjugation systems. This possibility is discussed at greater length in the final discussion (chapter 7).

1.7. The SOS response

This response is central to the experimental work described in this thesis and shall be considered in some detail.

1.7.1. A model for SOS regulation.

Exposure of *E. coli* to agents that damage DNA or interfere with DNA replication results in the induction of a diverse set of physiological responses collectively termed the SOS response (for a review see Walker, 1987). It is the largest and most complex DNA-damage inducible system to be characterized in detail in any organism. The physiological responses induced include an enhanced capacity for DNA repair, recombination and mutagenesis and are brought about by the increased expression of a set of genes often referred to as *din* (*d*amage *in*ducible) genes (Kenyon and Walker, 1980). These *din* genes are dispersed around the *E. coli* chromosome as well as on certain plasmids (Walker, 1987). The regulation of expression of these genes is controlled by the action of two proteins, RecA and LexA. In an uninduced cell, LexA acts as a repressor of unlinked *din*

genes by binding to a common operator sequence 5′ to each gene. Purified LexA protein has been shown to inhibit transcription *in vitro* and to bind to the operator sequence of a number of SOS genes (Walker, 1984). The consensus LexA binding site (referred to as a LexA or SOS box) is :

TACTGATATA-A-ACAGTA

Even though LexA binds to this site, many of the SOS genes are expressed at a significant level in the absence of SOS-inducing treatments. This may reflect differences in binding site affinity for the LexA repressor (Brent and Ptashne, 1981).

An inducing signal is generated when the cells DNA is damaged or replication is inhibited by certain temperature sensitive mutations. This inducing signal reversibly activates the RecA protein (RecA to RecA*) to act as a coprotease facilitating the proteolytic cleavage of LexA. This cleavage occurs at an -Ala-Gly- peptide bond near the middle of the polypeptide to generate two fragments (Horri *et al.*, 1981). Cleavage of the repressor is thought to result from a RecA-promoted autoproteolytic activity rather than a protease activity of RecA itself, since under conditions of high pH, specific cleavage of LexA can occur in the absence of RecA (Little, 1984). Other repressors such as the λ, P22 and 434 repressors are also cleaved at an -Ala-Gly- bond by the action of RecA* and therefore SOS treatments will result in the induction of bacteriophage lysogens.

Thus, after an SOS inducing treatment the level of functional LexA in a cell decreases and the SOS genes begin to be expressed at an increased level. Genes with operators that bind LexA weakly are the first to be fully turned on and *vice versa*. The *recA* and *lexA* genes themselves are members of the SOS regulon and therefore control their own expression. If the inducing signal is maintained, then more molecules of RecA are synthesized and subsequently activated and more molecules of LexA are cleaved. With a

sufficiently strong signal even genes whose operators bind LexA tightly will be maximally expressed (Walker, 1987).

When the inducing signal is removed, and the RecA molecules are no longer in the activated state, the continued synthesis of LexA results in a return to the repressed preinduction state.

1.7.2. Genetic studies on SOS induction.

Early genetic studies of the SOS regulon concerned mutants that prevented induction of the SOS responses (Howard-Flanders and Theriot, 1966; Mount *et al.*, 1972; Witkin, 1976). These generally fell into two classes, *recA*(Def) and *lexA*(Ind$^-$) mutants. The *recA*(Def) mutation was recessive and so indicated that RecA has a positive acting role, whereas the *lexA*(Ind$^-$) mutation was dominant suggesting the role of a repressor (Walker, 1987).

Roberts and Roberts (1975) studied the cleavage of bacteriophage λ repressor during SOS inducing treatments. The cleavage of λ repressor which occurred following treatment of a λ lysogen with ultraviolet (UV) light or mitomycin C (both powerful DNA damaging agents), correlated with expression of the phage genes. This λ induction and induced cleavage of the λ repressor was blocked in *recA*(Def) mutants suggesting that RecA is either a protease or regulates the production or activity of a protease.

A protein referred to as protein X, which later turned out to be the RecA protein, was shown to be induced under SOS inducing conditions. The induction of this protein was blocked by *lexA*(Ind$^-$) and *recA*(Def) mutants (Gudas, 1976). It was proposed that LexA repressed the gene coding for protein X and possibly other SOS genes and that RecA was involved in the inactivation of LexA (Gudas and Pardee, 1975). McEntee (1977), showed that protein X was in fact RecA and that it controlled its own induction.

The protein required for cleavage of the λ repressor was shown to be a product of the *recA* gene (Roberts *et al.*, 1977; Roberts *et al.*, 1978). As the

RecA protein was capable of mediating the cleavage of a repressor and the genetic studies indicated that RecA was involved in the inactivation of LexA, it seemed likely that LexA protein is cleaved in a RecA-dependent fashion at the time of SOS induction. Once purified LexA was available, it was shown to be cleaved *in vitro* in a similar way to the cleavage of λ repressor (Little, 1984; Slilaty and Little, 1987) and also that it acts as the direct repressor of *recA* and *lexA* genes.

1.7.3. Identification of genes in the SOS regulatory network.

The work described in Chapter 4 concerns the identification of genes, through the use of transcriptional fusions to *lacZ*, which are inducible by DNA damage. The identification and analysis of the regulation of the classical SOS genes was complicated due to the heterogeneous and complex physiological functions they specified. Kenyon and Walker (1980) used a Mu d1 (Ap *lac*) bacteriophage to construct operon fusions *in vivo*. They screened random Mu d1 (Ap *lac*) fusions in the *E.coli* chromosome for those that specified higher levels of β-galactosidase following treatment by mitomycin C. Using this tool, a set of damage inducible (*din*) loci were identified, whose expression was increased by SOS inducing treatments and blocked by *recA*(Def) and *lexA*(Ind$^-$) mutations. By this method, a number of genes were shown to be members of the SOS regulon, including *uvrA* and *uvrB* (Kenyon and Walker, 1981), *sfiA* (Huisman and D'Ari, 1981), *recA* (Casaregola *et al.*, 1982) and *umuDC* (Bagg *et al.*, 1981).

1.7.4. Nature of the SOS-inducing signal.

A major source of controversy concerning the SOS response is the nature of the SOS inducing signal that activates the coprotease function of RecA. The activation of RecA is reversible, which suggests that there may be a conformational change involved. *In vitro* studies have shown that

RecA becomes activated for coprotease function when it forms a ternary complex with single-stranded DNA and a nucleoside triphosphate (Craig and Roberts, 1980; Phizicky and Roberts, 1981). Thus it seemed possible that the single-stranded regions of DNA generated by SOS-inducing treatments form part of the *in vivo* signal. Indirect induction of the SOS system occurs upon the introduction of a UV-damaged DNA replicon such as ColIb, F, F', M13, λ, or λ derivatives carrying part of the F replication system, or Hfr DNA (D'Ari and Huisman, 1982; George *et al.*, 1974; Monk, 1969; Moreau *et al.*, 1982; Roberts and Devoret, 1983; Sommer *et al.*, 1985). This suggested that RecA is activated when it binds to gaps formed by the replication fork encountering a lesion.

SOS functions are induced by temperature shifts in *dnaB* (Ts) and *dnaE* (Ts) strains, consistent with the proposal that a stalled replication fork acts as an inducing signal (Schuster *et al.*, 1973; Witkin, 1976). Strains carrying a *recA441* mutation show constitutive expression of SOS responses at 42°C. It was proposed that the RecA441 (tif) protein becomes active due to an ability to bind to single-stranded regions of DNA that normally exist in the cell, such as those at the replication fork (Castellazi *et al.*, 1972; McEntee and Weinstock, 1981; Roberts *et al.*, 1982). However more recent data may indicate that the situation is actually more complicated and there is more than one pathway to SOS induction.

The *recBC* genes are required for induction of RecA protein synthesis by nalidixic acid but not ultraviolet light. The target of the antibacterial agent, nalidixic acid is the DNA gyrase enzyme (Gellert *et al.*, 1977; Sugino *et al.*, 1977) which causes double stranded breaks in DNA as an aberrant activity of the enzyme-drug complex. The single-stranded DNA produced by the unwinding action of RecBCD on such a substrate may act as the signal for RecA activation (Gudas and Pardee, 1975; Chaudry and Smith, 1985).

However, in contrast to nalidixic acid, most inducing agents do not break DNA but instead make lesions that change the structure of the bases.

Sassanfar and Roberts (1990), in agreement with the earlier work involving indirect induction, suggest that the major pathway of induction after damage by a typical agent such as UV light requires an active replication fork. UV induced lesions can be repaired by excision repair (Sancar and Rupp, 1983). In the absence of repair, DNA synthesis will become discontinuous and gaps will occur (Rupp and Howard-Flanders, 1968). Thus, RecA may bind to the ssDNA that is left when the replication fork encounters non-coding lesions. The inducing signal following UV-irradiation requires replication whereas agents that lead to DNA breaks directly such as the drug bleomycin or nalidixic acid acting on gyrase do not (Sassanfar and Roberts, 1990). Salles and Defais (1984) showed that RecA protein induction by UV is blocked if initiation of DNA replication is prevented by a temperature-sensitive mutation. Blocking the elongation stage in DNA replication by drugs or mutation prevents or slows LexA cleavage.

There are situations where temperature-sensitive DNA elongation mutants both stop the replication fork and induce LexA cleavage at varying rates at the non-permissive temperature (Schuster *et al.*, 1973). Rather than the inducing signal being due to a stalled replication fork, the signal may result from affected proteins dissociating from the fork leaving regions of exposed single-stranded DNA. For example a situation where *dnaE* activity is lost but the activity of primase (*dnaG*) and helicase (*dnaB*) remains may result in unwinding of DNA and regions of ssDNA.

Thus, there is strong evidence to suggest that regions of single-stranded DNA can act as the inducing signal for triggering the SOS responses and that replication is required for induction following a lesion-introducing treatment. However, there are indications that there may be a

second pathway to SOS induction, that being the binding of RecA directly to damaged duplex DNA. Lu and Echols (1987) showed there to be a correlation between the ability of wild type and mutant RecA proteins to promote SOS-induced mutagenesis and this capacity to bind to double-stranded DNA. Wild type RecA binds more efficiently to UV irradiated duplex DNA than to non-irradiated DNA. The RecA441 (Tif) protein binds very well to duplex DNA with no lesions, whereas RecA430 which is defective in mutagenesis binds poorly even to UV-irradiated duplex DNA. The RecA phenotype also correlates with the capacity to use duplex DNA as a cofactor for cleavage of the LexA repressor. This provides an alternative pathway for SOS induction but it is unclear if the binding of RecA to UV induced lesions is an important signal *in vivo*.

1.7.5. Naturally occurring plasmids carry SOS inducible genes.

It may be relevant to this work that certain plasmids carry SOS inducible genes. One of the genes studied in this work is *psiB*, which encodes a protein capable of interfering with the SOS response of the host cell. It is located on the ColIb plasmid, which carries three known SOS inducible loci, *imp* (I group mutation and protection), *cib* (Colicin Ib) and *abi* (abortive phage infection). The *cib* and *abi* genes were described in section 1.6.1. This section shall describe *imp* and other similar plasmid loci which can substitute for *E. coli umuCD* mutations.

In *E. coli*, mutagenesis by certain DNA damaging agents is not a passive process but actually requires the intervention of a system that processes damaged DNA in a mutagenic manner. It requires the products of at least three genes, *umuD*, *umuC*, and *recA* which are all inducible as members of the SOS regulon. Mutations in *umuD* or *umuC* abolish the ability of *E. coli* cells to be mutated by agents such as UV and 4-nitroquinoline-1-oxide (Elledge and Walker, 1983a; Kato and Shinoura, 1977;

Shinagawa *et al.*, 1983). Such mutant strains are still capable of expressing a wide variety of SOS responses such as induction of λ prophage. The *umuD* and *umuC* genes are located in an operon and specify products of 16 kd and 45 kd. Through the use of a Mu d 1 (Ap *lac*) insert in *umuC*, Bagg *et al* (1981) showed that *umuC* was induced by SOS inducing treatments and was regulated by *recA* and *lexA*. SOS mutagenesis results in mostly targetted mutations (ie mutations occur at or near the original site of lesion) and is thought to be due to a relaxation of the fidelity of DNA polymerase permitting DNA synthesis and misincorporation of nucleotides opposite lesions in the template strand (Walker, 1984). Activated RecA protein is required initially for the derepression of the SOS regulon to allow expression of *umuDC* but is also thought to have a two additional roles in the process of mutagenesis (Bagg *et al.*, 1981; Blanco *et al.*, 1982; Ennis *et al.*, 1985). It has been shown that UmuD protein is cleaved after a mutagenic treatment in a RecA* dependent manner (Burckhardt *et al.*, 1988; Shinagawa *et al.*, 1988). The UmuD protein contains a sequence similar to that surrounding the cleavage sites of the λ, P22, 434 and LexA repressors and there is RecA* mediated cleavage of UmuD at its bond between Cys-24 and Gly-25 (Nohmi *et al.*, 1988; Perry *et al.*, 1985).

The third role for RecA protein in the mutagenic process is more direct and independent of its regulatory function (Ennis *et al.*, 1985; Witkin and Kogoma, 1984; Lu *et al.*, 1986). It has been shown that RecA binds preferentially to the site of DNA damage (eg UV induced pyrimidine dimers) and is able to inhibit the editing subunit (ε) of DNA polymerase III. Most SOS-induced mutagenesis occurs during replication across a DNA lesion and so it is proposed that RecA* coats the lesion and allows replication across with reduced fidelity. The role of UmuDC is as yet unclear in this model. It is suggested that UmuDC allows DNA strand elongation past the lesion once bases have been misincorporated (Bridges and

Woodgate, 1984; Christensen *et al.*, 1988; Tessman, 1985). An alternative explanation is that UmuDC facilitates the binding of RecA at the site of lesion.

The presence of certain plasmids can suppress the nonmutability of *umuDC* mutants and increase the resistance of *E. coli* and *S. typhimurium* to killing by UV irradiation (Shanabruch and Walker, 1980; Walker and Dobson, 1979; Sedgwick *et al.*, 1989). This effect was first observed for the archetypal IncI1 plasmid, ColIb-P9 (Howarth, 1965; 1966). The best studied of the plasmids that have the *umuDC* suppressing ability are pKM101 and TP110. The interest in pKM101 arose due to its inclusion in the Ames mutagenesis tests as the plasmid makes the bacterial tester strain more mutable (McCann *et al.*, 1975). pKM101 is a deletion derivative of R46 (IncN) which carries a locus termed *mucAB*. TP110 is a kanamycin resistant derivative of ColIb (Dowden *et al.*, 1984) and the genes responsible for *umuDC* suppression are referred to as *impCAB* (I group mutation and protection) genes. These plasmid loci are regulated as part of the SOS response and apart from their common regulation, also have considerable sequence similarity with the *E. coli umuCD* operon (Glazebrook *et al.*, 1986; Lodwick *et al.*, 1990; Perry and Walker, 1982; Perry *et al.*, 1985; Sugino *et al.*, 1977).

The MucA and ImpA proteins share ~41 % identity with UmuD. ImpA and MucA are cleaved in a RecA dependent manner at an Ala-Gly bond, whereas UmuD has a Cys-Gly cleavage site. A major difference between the three loci is the presence in the *imp* operon of a third gene, *impC*. The product of this gene is a small protein (9.4 kD) which has no known function and is not required for the UV protection and mutation phenotype (Lodwick *et al.*, 1990).

An analysis of conjugative plasmids from eight incompatibility groups from the Murray collection of 'pre-antibiotic era' enterobacteria

identified IncI1 and IncB plasmids which restored ultraviolet resistance and induced mutability to *umuC* mutants. These plasmids also increased the UV resistance and mutability of wild type *E. coli, Klebsiella* and *Citrobacter* and carried sequences similar to the *impCAB* locus of modern day IncI1 plasmids (Sedgwick *et al.*, 1989). Similarly the *mucAB* genes of pKM101 caused an increase in UV resistance of species of enterobacteria that have poor or nonexistent mutagenic DNA repair systems (Sedgwick and Goodwin, 1985). These results suggest that mutagenic DNA repair genes are long standing plasmid features and may confer a selective advantage on their hosts. It is interesting that the *imp* locus exists on plasmids which also carries the gene, *psiB*, which can interfere with the SOS response of the host.

1.7. The leading region.

The two genes central to this work, namely *psiB* and *ssb* are located within the leading region of the plasmid. The *oriT* site on many different conjugative plasmids maps at or near one end of the Tra region. This arrangement is found on F-like plasmids (Ippen-Ihler and Minkley, 1986; Willetts and Skurray, 1987), IncI1 plasmids (Rees *et al.*, 1987), IncN (Winans and Walker, 1985) and IncP (Lanka and Barth, 1981). All plasmids studied thus far transfer their DNA in a preferred direction from *oriT*, with most, if not all of the tra genes entering the recipient cell last (Al-Doori *et al.*, 1982; Grinter, 1981; Howland and Wilkins, 1988; Willetts and Skurray, 1987). This pattern of *tra* gene transfer may be significant and the late transfer of the *tra* determinants be important either to maintain their expression in the donor cell or to delay their expression in the newly infected recipient until the DNA transport process is completed.

The leading region of a conjugative plasmid is the segment first to enter the recipient cell during transfer. This part of the F plasmid is defined as the 13 kb between *oriT* at 66.7F and the primary replication region,

RepFIA. Genes identified in this region for which a defined phenotype has been described are *flm*, specifying a plasmid maintenance system, *psiB*, determining plasmid SOS inhibition and *ssb*, which encodes a single-stranded DNA binding protein (Kolodkin *et al.*, 1983; Bagdasarian *et al.*, 1986; Golub *et al.*, 1988; Loh *et al.*, 1988). The *psiB* and *ssb* genes are described in greater detail in the following sections. It is interesting that plasmids which specify quite distinct conjugation systems have significant homologies to the leading region of F (Golub and Low, 1986). Plasmids from the F and I complex of incompatibility groups carry homologous *ssb* and *psiB* genes, even though the respective conjugation systems do not appear to show any similarities (Howland *et al.*, 1989; this work).

1.8. Plasmid-encoded SOS inhibition.

In vivo studies of SOS induction indicated that stretches of single-stranded DNA can act as a powerful SOS inducing signal (section 1.7.4). Conjugation involves the transfer of single-stranded DNA from donor to recipient cell. Thus, it is possible that this process of single-stranded DNA transfer may act as a trigger for the SOS response. A plasmid locus termed *psi* (plasmid SOS inhibition) was identified on plasmids R6-5 and R100 and shown to inhibit induction of cellular SOS genes and prophage lambda. The Psi tests followed prophage induction in a *recA441* λ lysogen (*recA441* strains express SOS functions at 42°C) or *sfiA::lacZ* expression in *recA441* and *recA730* (constitutive SOS functions) backgrounds. The *sfiA* gene is a member of the SOS regulon under *recA/lexA* control.

Initial observations showed that the presence of R100 inhibited prophage λ induction and *sfiA::lacZ* expression (Bagdasarian *et al.*, 1980), as did R6-5 (Bagdasarian *et al.*, 1986). Subcloning from R6-5 located the locus responsible for the phenotype to an 8.1 kb fragment in the leading region of the plasmid. The psi locus was located more precisely through the use of

Tn*3* transposon insertions that inactivated Psi function. Such Tn*3* insertions reduced the synthesis of two polypeptides of sizes 24.5 kD and 12.5 kD, designated PsiA and PsiB respectively. Deletion analysis of this region showed the genes specifying these polypeptides to be transcribed in the order *psiB* to *psiA* towards the *oriT* site (Bailone *et al.*, 1988). However, plasmids expressing *psiB* alone were able to confer a Psi$^+$ phenotype, indicating that *psiA* does not affect Psi function. The role of *psiA* is as yet undefined (Loh *et al.*, 1990).

The mechanism of how PsiB exerts its inhibitory role has been studied primarily by genetic methods. The potential stages for PsiB activity are at the level of RecA activation, LexA cleavage or SOS gene expression. A *psiB*$^+$ plasmid failed to reduce the high level of *sfiA* expression in a host devoid of active LexA. Thus, the Psi function is apparently not due to a reduction of SOS gene expression by PsiB acting as an analogue of LexA (Bagdasarian *et al.*, 1986). Another possibility is that LexA cleavage is prevented due to an interaction between PsiB and LexA. However, the SOS inhibition appears to vary as a function of the *recA* allele used and therefore implies that the interaction is with RecA not LexA. MucA and ImpA have regions of homology with LexA and can inhibit SOS induction presumably by a competitive mechanism (Marsh and Walker, 1987; Strike and Lodwick, 1988). However, there is no evidence of homology between PsiB and LexA.

The high level of spontaneous mutations observed in *recA730* and *recA441* strains in the absence of DNA damage is thought to be due to the increased expression of *umuDC* and a readily activated RecA protein. It is possible to measure this mutator phenotype by looking at the reversion rate of a mutation such as *his-4*. Bailone *et al.* (1988) showed that PsiB inhibits the mutator phenotype of *recA441* or *recA730* cells, implying that PsiB affects the RecA coprotease activity on the LexA repressor, UmuD or both (Shinagawa *et al.*, 1988; Nohmi *et al.*, 1988). PsiB has been shown to inhibit

in vivo the RecA* promoted cleavage of both LexA and UmuD (Adrianne Bailone, personal communication). Thus LexA does not appear to be the direct target of PsiB but RecA may be. PsiB also has an inhibitory effect upon RecA activity in recombination. There is an inhibitory effect for intra-chromosomal recombination but not homologous recombination following conjugation (Bailone *et al.*, 1988). Presumably the difference reflects the different DNA substrates involved in these two events.

The level of PsiB specified by R6-5 is sufficient to inhibit *sfiA* induction in unirradiated *recA730* or *recA441* bacteria but this level was insufficient to prevent induction of *sfiA* after UV-irradiation of *recA*$^+$ bacteria. Only when *psiB* is under the control of an inducible promoter, such that the concentration of PsiB is amplified, is *sfiA* induction prevented in UV irradiated *recA*$^+$ cells (Bailone *et al.*, 1988). This result indicates that the inhibitory properties of PsiB depends on its concentration and again suggests that a direct interaction with RecA protein may be involved.

PsiB shows no ability to bind to single-stranded DNA through the use of a binding column and maxi-cell extracts. Thus it is unlikely that the inhibitory effect is due to PsiB competing with RecA for single-stranded DNA regions (Bailone *et al.*, 1988). Hence, many possible mechanisms to account for the inhibitory effect of PsiB have been ruled out. The most likely mechanism for the action of PsiB remains one involving prevention of RecA activation. RecA mutants have been recently been isolated that are resistant to the inhibitory effect of PsiB (Adrianne Bailone, personal communication). It is possible that there is a direct interaction between the two proteins which prevents RecA reaching the activated form possibly through steric hindrance.

A gene homologous to *psiB* of R6-5 is carried by plasmid F (Golub *et al.*, 1988). However unlike R6-5, F fails to express the PsiB function unless the *psiB* gene is cloned in a multicopy form. When carried on a multicopy

vector, the F *psiB* gene is as effective as the corresponding R6-5 *psiB* gene at conferring a Psi phenotype. A comparison of the predicted amino acid sequences of the two polypeptides reveals only 5 differences in the 144 residues (Dutreix *et al.*, 1988). Four of these changes are in the carboxy terminal region which is thought to be outside of the active centre of the protein as it can be deleted without loss of function (Bailone *et al.*, 1988). Thus it is unlikely that the F PsiB is intrinsically less active than the R6-5 PsiB. Another possible explanation is that the concentration of PsiB in F containing cells is naturally lower than that in cells harbouring R6-5. The expression of the two genes was compared through the use of transcriptional fusions to *lacZ* in multicopy vectors and it was found that R6-5 *psiB* expression was four times higher than that of F. Wild-type R6-5, and R100 which also expresses a constitutive Psi phenotype, carries a copy of the Tn*10* transposon located upstream of *psiB*, which is absent from F. It is known that Tn*10* can, under certain circumstances, provide a promoter for adjacent genes (Ciampi *et al.*, 1982). By deleting the Tn*10* IS10-R p-OUT promoter, expression of the R6-5 *psiB::lacZ* fusion is reduced to a level equivalent to that of F *psiB*. This indicates that the strong Tn*10* p-OUT promoter is responsible for the enhanced expression in R6-5. Random Tn*10* insertions have been isolated in F and tested for those that trigger constitutive Psi function. Six independently isolated Psi^+ mutants were generated and all found to be located upstream of *psiB*. Four were in *ssb*, one upstream of *ssb* and one between the two genes (Adrianne Bailone, personal communication).

Recently R6-5 PsiB polypeptide has been purified and N-terminal sequencing revealed that the first 13 amino acids match that predicted from the nucleotide sequencing. The native state of PsiB is a tetramer and the concentration of PsiB in R6-5 containing cells is ~1500 tetramers per cell. The number of PsiB molecules increases in proportion to the number of

psiB gene copies in the cell, so there is no evidence of negative autoregulation of *psiB* by its product. In extracts of established F containing cells, PsiB is undetectable by immunoblotting emphasizing that the concentration of F PsiB is naturally lower than R6-5 PsiB in established strains (Mira Bagdasarian, personal communication).

1.9. Single-stranded DNA binding proteins.

The single-stranded DNA-binding protein (SSB) of *E. coli* plays a central role in the cell by participating in a variety of DNA metabolic processes such as replication, repair and recombination. Plasmids from a wide range of incompatibility groups specify SSBs with striking similarity to *E. coli* SSB and are able to suppress the temperature sensitivity of chromosomal *ssb* mutations indicating that the proteins are functionally interchangeable. For all plasmids examined so far, the SSB protein is encoded by a gene in the leading region and is highly conserved even though the conjugation systems specified by these plasmids are quite distinct. In order to consider the possible role of these plasmid SSB proteins it may be helpful first to consider the properties of the chromosome-encoded protein.

1.9.1. The single-stranded DNA-binding protein of *E.coli*

E. coli SSB readily binds to single-stranded DNA in a manner that is cooperative and lacks sequence specificity (Schneider and Wetmur, 1982). In its native form the protein exists as a homotetramer of 18.8 kD subunits (Sancar *et al.*, 1981; Williams *et al.*, 1983). Both the nucleotide and amino acid sequences have been determined and show the protein to consist of 177 amino acids with the initiating methionine absent. The amino terminal domain (1 - 105) contains most of the charged residues and is predicted to have a highly ordered structure consisting of α helix and β pleated sheet.

Proteolytic studies have indicated that the amino terminal region contains the DNA binding domain and that a triple β-sheet motif be the essential component (Williams *et al.*, 1983). In the region between residue 106 and 165 there are very few charged amino acids and the structure is predicted to be random coil and β bends. It is possible that this region towards the carboxy terminus is important for interaction with other proteins (Chase and Williams, 1986).

The two best characterised *E. coli ssb* mutations are *ssb-1* (Glassberg *et al.*, 1979; Sevastopoulos *et al.*, 1977) and *ssb-113* (Greenberg *et al.*, 1974). Both show temperature sensitive lethality at 44°C due to rapid cessation of replication at high temperature. At a temperature of 30°C, *ssb-1* mutant strains are phenotypically normal except for a slight UV sensitivity (Lieberman and Witkin, 1981), whereas *ssb-113* strains show other defects also.

1.9.2. The multiple roles of *E. coli* SSB.

There is an absolute requirement for SSB in replication, recombination and repair. The essential role of SSB in DNA replication was unequivocally established through the use of *ssb* mutants. As mentioned above, strains carrying the *ssb-1* mutation are temperature sensitive for DNA synthesis *in vivo* (Glassberg *et al.*, 1979). Replication terminates within one to two minutes of the temperature shift indicating that *ssb-1* is a quick stop mutation and that SSB is probably involved in chain elongation. SSB is thought to have multiple roles in replication: (i) it enhances helix destabilization by helicases; (ii) prevents reannealing of the single-strands and protects against single-strand nucleases; (iii) helps to stabilise and organise replication origins; (iv) is required for priming; (v) ensures the specificity of priming; (vi) enhances the fidelity of DNA polymerase; (vii) enhances the processivity of the polymerase by destabilising secondary

structure that could cause pausing and disassociation; and (viii) it may promote binding of the polymerase to the template (for a review of these processes see Meyer and Laine, 1990).

The first evidence that SSB is involved in DNA repair processes came from the observation that *ssb* mutants are UV sensitive (Glassberg *et al.*, 1979; Lierberman and Witkin, 1983). Subsequent studied have provided evidence that SSB plays a role in methyl-directed mismatch repair, the SOS response and recombinational repair. *In vitro,* mismatch repair has an absolute requirement for SSB (Lahue *et al.*, 1989; Lu *et al.*, 1984). Three key enzymes in the process can interact functionally with SSB: DNA helicase II unwinding is driven by SSB, exonuclease I is stimulated by SSB to cause the exonucleolytic excision of the displaced error-containing strand, and repair synthesis by DNA polymerase III requires SSB to be bound to the template strand.

SSB is known to promote several RecA-mediated reactions. The key step in the triggering of the SOS response is the activation of RecA protein (see section 1.7.). Strains carrying *ssb* mutations are defective in SOS induction and SSB is thought to be involved in the activation of RecA (Cohen *et al.*, 1983; Meyer *et al.*, 1982; Whittier and Chase, 1981, 1983). The proteolytic activity of RecA under conditions of excess ssDNA is stimulated by SSB (Weinstock and McEntee, 1981) and it has been shown that SSB increases both the rate and extent of RecA-promoted strand assimilation reaction (McEntee *et al.*, 1980; West *et al.*, 1982). These effects may be due to the removal of secondary structure thereby removing an impediment to RecA protein binding (Tsang *et al.*, 1985). Flory and Radding (1982) used electron micrographic techniques to show that SSB increased the formation of extended filaments of single-stranded DNA and RecA protein by more than 50 fold. Kowalezykowski and Krupp (1987) suggested the possibility

that RecA protein may initially only be able to bind to regions of ssDNA devoid of secondary structure.

There is much contradictory data concerning the effect of overproduction of SSB on these essential cellular processes. *In vitro* studies by Cohen *et al.* (1983) indicated that whereas excess SSB was able to inhibit RecA promoted ATPase activity, the binding of SSB could not completely eliminate the binding of RecA to ssDNA. RecA coprotease activity could not be eliminated completely by SSB. Overproduction of SSB *in vivo* inhibits some UV induced RecA-dependent processes (recombinational repair) and results in a mild increase in UV sensitivity However, it only has a small effect on LexA and λ repressor cleavage (Moreau, 1987; 1988). It is suggested that, whereas the formation of long nucleoprotein filaments required for the initiation of recombination is inhibited by excess SSB, the formation of short tracts of RecA required for repressor cleavage is not.

1.9.3. Regulation of *E. coli ssb* expression.

The *ssb* gene of *E. coli* lies adjacent to the inducible *uvrA* gene (Sancar *et al.*, 1981) and the *uvrA* and *ssb* genes are transcribed in opposite directions (Brandsma *et al.*, 1983). The -35 sequence of *uvrA* overlaps a LexA box (Backendorf *et al.*, 1983) and there is conflicting evidence whether or not the *ssb* gene is itself subject to SOS regulation. Alazard (1983), Salles *et al.* (1983), Whittier and Chase (1981), and Villani *et al.* (1984) all failed to show that the *ssb* gene is inducible. However, Brandsma *et al.* (1983) used a fusion between the *galK* structural gene and *ssb* and found there to be an increase in galactokinase levels following DNA damage. The *ssb* gene is believed to have three promoters that function *in vivo* (Brandsma *et al.*, 1985). Two of these promoters, designated PN1 and PN2 are not inducible and appear to function equally well to provide constitutive levels of SSB. Transcription from the third promoter, termed PI, was shown to be inducible. The -35

sequence of this inducible promoter lies only seven nucleotides from the -35 sequence of the *uvrA* and only two nucleotides from the upstream limit of the LexA repressor recognition sequence. An examination of *in vivo* transcripts before and after SOS induction indicates that efficient transcription from the PI promoter is accompanied by a dramatic decrease in transcription from promoters PN1 and PN2. However, using a radioimmune assay (Paoletti *et al.*, 1985), various groups have failed to detect any amplification of SSB protein under SOS inducing conditions (Salles *et al.*, 1983; Villani *et al.*, 1984). In a non-inducible *lexA3* strain, the LexA repressor inhibits *uvrA* induction but does not have any effect on the level of *ssb* expression (Alazard, 1983). These apparent contradictions may be explained by the fact that Brandsma and co-workers did not measure the levels of SSB directly but measured the activity levels of a reporter gene. Perrino *et al.* (1987) showed that there was an increase in the relative rate of synthesis of SSB after an SOS inducing treatments but there was no detectable increase in the level of accumulated SSB. Moreau (1987) reported a twofold increase in SSB levels, but only after a three hour exposure to high levels of mitomycin C. Thus, although it appears that *ssb* may be inducible, induction is low relative to that of other genes in the *recA-lexA* SOS regulon (Walker, 1984; 1985) and it is unclear as to its significance.

1.9.4. Plasmid-encoded single-stranded DNA binding proteins.

Kolodkin *et al.* (1983) showed that the introduction of plasmid F resulted in the partial suppression of the temperature sensitivity of *ssb-1* and *ssb-113* mutant strains and the suppression was complete if the appropriate region of the F plasmid was cloned onto a high copy number vector plasmid. The suppressing activity is due to the expression of a protein product termed F SSB, which binds to single-stranded DNA under similar conditions to *E. coli* SSB. The gene responsible is designated F *ssb*

and maps on the E3 (*EcoRI*) fragment of the F factor at map position 55.2 (Cram *et al.*, 1984). This location is within the leading region of the plasmid. F SSB has 178 amino acid residues, which is one more than *E. coli* SSB, and has a molecular weight of 19,505. There is extensive sequence similarity between the SSBs of *E. coli* and plasmid F, which is particularly evident in the amino terminal region where 87 of the first 115 residues are identical. This is the region thought to be important for DNA binding (see section 1.10.1).

The ability of conjugative plasmids from a wide range of incompatibility groups to suppress defects in the chromosomal *ssb* gene was investigated (Golub and Low, 1985). It was shown that representatives from 12 out of 23 incompatibility groups were able to suppress *ssb-1* mutations and, using Southern hybridization that these plasmids carried sequences homologous to the F *ssb* gene. These plasmid *ssb* genes were more similar to each other than to the *E. coli ssb*. The plasmids also showed homology to other sequences in the F leading region, suggesting that a larger module might be conserved as a unit (Golub and Low, 1986b).

Plasmids carrying mutations that derepressed the genes involved in the conjugative transfer process showed a higher level of *ssb-1* suppression than the parental repressed plasmids, indicating that plasmid *ssb* genes are coregulated as part of the transfer system (Golub and Low, 1986a; Howland *et al.*, 1989). One implication of this result is that plasmid SSB has a role in conjugation and possibly in conjugative DNA metabolism. The *ssb-1* strains at the permissive temperature are slightly UV sensitive. Presence of a ssb^+ plasmid will suppress this sensitivity even when the fertility inhibition system is intact suggesting that the plasmid *ssb* gene is expressed at two levels: a constant low-level expression which might be sufficient for suppression of the UV sensitivity, whereas higher level expression might be required for suppression of temperature sensitivity, with this state being

achieved when the transfer system is fully expressed (Golub and Low, 1986a; Howland et al., 1989).

The IncI1 plasmid ColIb-P9 carries an *ssb* gene with 84% nucleotide sequence identity to F *ssb* (Howland *et al.*, 1989). Moreover, it is located in the leading region, 11 kb from *oriT* in an equivalent position to the *ssb* determinant on F. The direction of transfer of ColIb, relative to the *tra* genes, is the same as that established for F (Howland and Wilkins, 1988). The similarities between the sequences and positions of the ColIb and F *ssb* genes are interesting since the plasmids belong to incompatibility groups which share very little DNA homolgy, and the respective conjugation systems are judged to be distinct (Falklow *et al.*, 1974; Grindley *et al.*, 1973; Rees *et al.*, 1987.

A mutant of ColIb*drd* carrying a Tn*903*-derived insertion in *ssb* was constructed, but was unaffected in the ability to generate plasmid transconjugants and was maintained stably in donor cells both following conjugation and during vegetative growth (Howland *et al.*, 1989). However, unlike the parental plasmid, such ColIb *ssb* mutants conferred a marked Psi$^+$ phenotype on the host strain. Plasmids F and R6-5 have a *psi* locus downstream of the *ssb* gene. Therefore it was suggested that the Psi$^+$ phenotype, conferred by the ColIb *ssb* mutant, was not due to the mutant ColIb SSB protein *per se* but was the consequence of altering the regulation of a *psi* gene.

1.10. Aims of this project.

The purpose of this work was to further the understanding of the function of the ColIb leading region. It is apparent that unrelated conjugative plasmids share considerable homologies in their leading regions and so this region may have an important role in plasmid biology.

The approach taken to investigate the leading region genes was to study the regulatory elements controlling their expression.

The initial aim of this project was to investigate the observation of Howland *et al.* (1989), that a ColIb *ssb* mutant conferred a Psi$^+$ phenotype on the host cell, as this result indicated that ColIb encodes a potentially active Psi protein. The thesis describes the identification and sequence analysis of a *psiB* gene on ColIb. Transcriptional fusions were created between *lacZ* and the *ssb* and *psiB* genes on ColIb and ColIb*drd-1*. These fusions were then used to study the regulation of these leading region genes. A major question was to determine whether the leading region genes are coordinately regulated as part of the ColIb transfer system. Therefore, as a control a *lacZ* transcriptional fusion was also made to *sog*, regarded here as a representative transfer gene.

Insertion mutants of ColIb *psiB* were constructed and used to test the hypothesis that PsiB prevents induction of the SOS response during the conjugative transfer of single-stranded DNA. The Appendix describes two further analyses which, although of relevance to the final discussion, did not merit inclusion in a results chapter.

Chapter 2.
The *psiB* gene of ColIb.

2.1. Introduction.

This chapter concerns the identification of a *psiB* gene on the IncI1 plasmid ColIb. As described in Chapter 1, studies on Psi function had centred on the *psiB* genes of plasmids F and R6-5. Sequences homologous to the R6-5 *psiB* gene had been identified on plasmids from nine distinct incompatibility groups including a representative of the IncI1 group, but the precise location and details were not examined (Golub *et al.*, 1988). Presence of a functional *psiB* gene on ColIb had previously been indicated by the finding that a mutant of ColIb*drd-1* carrying a Tn*903* based Km^r insertion in the *ssb* gene resulted in the production of a Psi^+ phenotype. The presence of the intact ColIb *ssb* gene on a multicopy vector did not affect the Psi^+ phenotype of the *ssb* mutant, suggesting that the effect was not due to the truncated SSB protein *per se* but was the consequence of the altered regulation of a *psi* gene (Howland *et al.*, 1989; Howland, 1989). The *psiB* gene of both F and R6-5 is located downstream of an *ssb* gene, so it might be expected that the ColIb *psiB* gene would be located similarly.

Two strategies were used to identify the *psiB* gene of ColIb. The first involved Southern hybridization against cloned fragments of ColIb, using a probe carrying most of the R6-5 *psiB* gene, whereas the second involved genetic tests of Psi activity. Psi function can conveniently be monitored by measuring the β-galactosidase activity specified by a *sfiA::lacZ* fusion in *recA441* or *recA730* backgrounds. SOS responses are expressed constitutively in *recA730* strains and at high temperature (42°C) in *recA441* strains. The *sfiA* gene is a member of the SOS regulon, which acts to block cell division allowing filamentation (Huisman and D'Ari, 1981). SOS induction results in the induction of a λ prophage due to RecA promoted cleavage of the cI

repressor (Walker, 1987). Thus, Psi function can also be monitored by observing cell lysis from a *recA441* λ lysogen at 42°C.

2.2. Locating the ColIb *psiB* gene.

To show that ColIb carries a gene homologous to the *psiB* gene of R6-5 and to determine its approximate location, a Southern hybridization experiment was carried out using a 0.5 kb *Eco*RV fragment of pGY7568 as a probe. This fragment contains ~80% of the R6-5 *psiB* coding sequence and lacks the first 60 bp (Bailone *et al.*, 1988; Dutreix *et al.*, 1988). Plasmid F is known to contain a *psiB* gene with considerable nucleotide sequence identity to that of R6-5 and so was included as a positive control (Dutreix *et al.*, 1988; Golub *et al.*, 1988). On these F-like plasmids, *psiB* is located in the leading region, downstream of an *ssb* gene. Thus, the ColIb-derived test plasmids were ColIb*drd-1*, pLG2001, pCRS3, and pLG283. Plasmid pLG2001 carries the E1 *Eco*RI fragment of ColIb, which contains both the *oriT* site and the *ssb* gene. Plasmids pCRS3 and pLG283 contain fragments from the ColIb leading region with pCRS3 carrying the *oriT* region and pCH1 the region including *ssb* (Fig. 2.1).

Figure 2.2 shows that the R6-5 *psiB* probe hybridised to an 11.4 kb *Eco*RI fragment of plasmid F (and to uncut F DNA), to a 10.1 kb *Sal*I fragment (S4) of ColIb*drd-1*, a 4.8 kb *Pst*I fragment of pLG2001 and a 3.9 kb *Pst*I fragment of pLG283. There was no hybridization to pCRS3. Thus the sequence showing homology to the R6-5 *psiB* probe is contained on a 2.0 kb *Sal*I-*Pst*I fragment between coordinates 22.1 and 24.1 on ColIb (Fig. 2.1). As the probe used did not contain the entire R6-5 *psiB* coding sequence, it could not be ruled out at this stage that the ColIb *psiB* gene extends over the *Sal*I or *Pst*I sites. From this initial data the ColIb *psiB* gene appears to be located downstream of the *ssb* gene in a corresponding position to the *psiB* genes of F and R6-5.

Fig. 2.1. Restriction maps of plasmids carrying ColIb *ssb* and the putative location of *psiB*.

Line 1 (top) indicates kilobase coordinates on the ColIb map (Rees *et al.*, 1987). Line 2 shows restriction sites in the E1 *Eco*RI fragment of ColIb, the location of the *ssb* gene and the origin of transfer (*oriT*). The direction of DNA transfer from *oriT* is from left to right. Transcription of *ssb* is from right to left. Line 3 shows plasmid pLG283 linearized at one end of the pBR328 vector (filled box). Broken lines between lines 2 and 3 indicate the extent of deletion present in pLG283. The thick black lines represent the restriction fragments which hybridized to the R6-5 *psiB* probe and the hatched box, the putative location of ColIb *psiB* . Restriction sites are *Acc*I (A), *Cla*I (C), *Eco*RI (E), *Pst*I (P), *Sal*I (S). pCRS3, which is not shown, contains ColIb DNA from coordinates 24.1 to 40.0.

Fig.2.1.

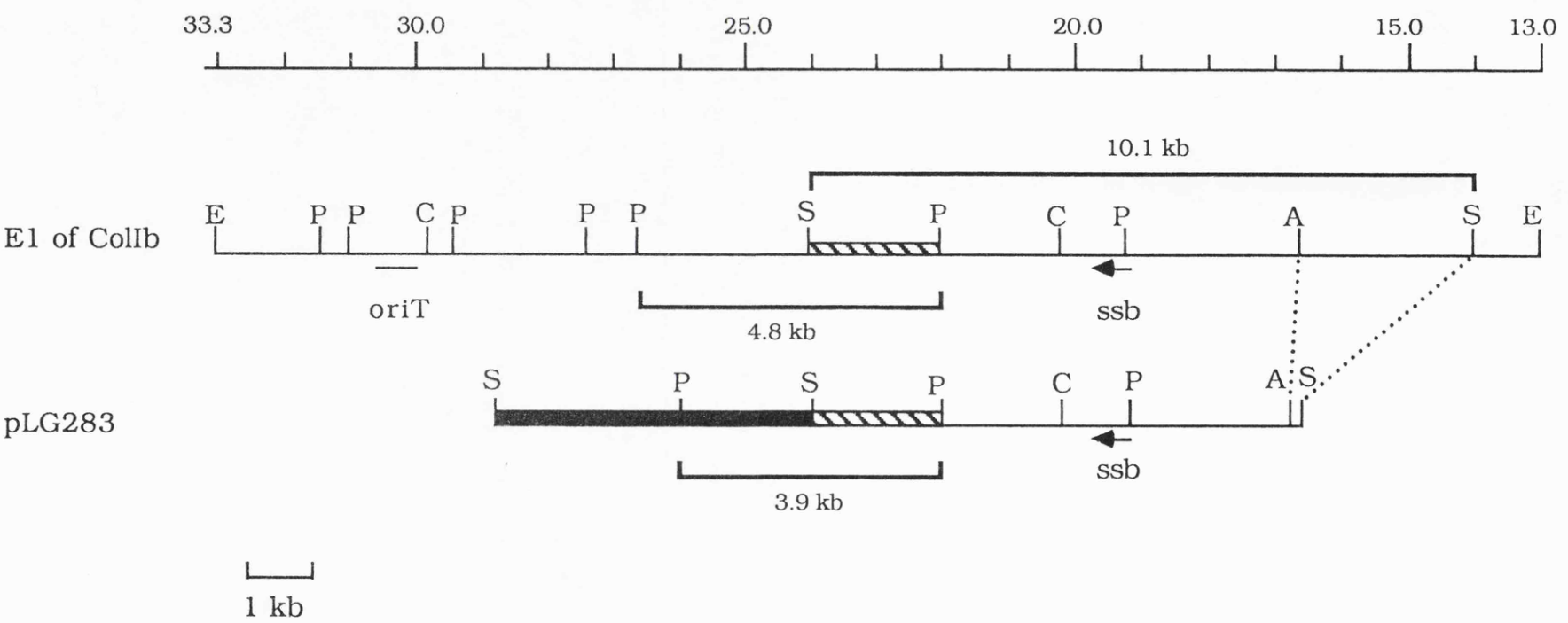
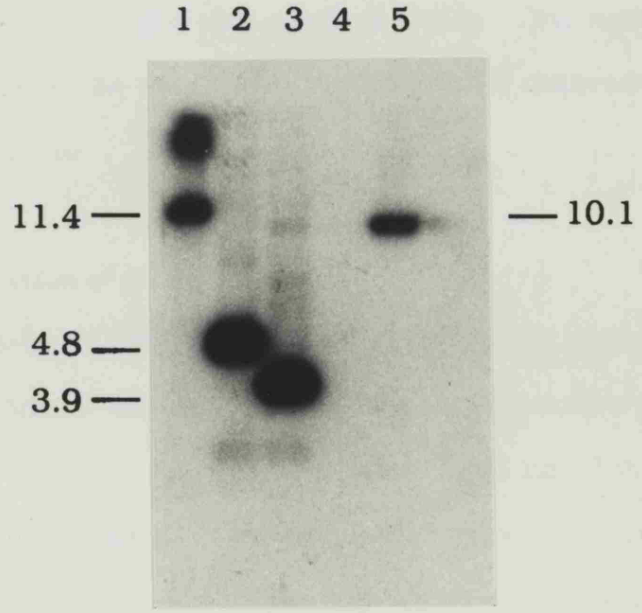

33.3
30.0
25.0
20.0
15.0
13.0
10.1 kb
E1 of ColIb
E P P C P P P S P C P A S E
oriT
4.8 kb
ssb
pLG283
S P S P C P A S
ssb
3.9 kb
1 kb

Fig 2.2. Results of a Southern hybridization of a 0.5 kb *Eco*RV fragment carrying R6-5 *psiB* to restriction fragments of F, ColIb*drd-1*, pLG2001, pCRS3 and pLG283.

Lanes correspond to (1) F x *Eco*RI, (2) pLG2001 x *Pst*I, (3) pLG283 x *Pst*I (4) pCRS3 x *Pst*I and (5) ColIb*drd-1* x *Sal*I separated by agarose gel electrophoresis. Autoradiography was for 1.5 hours.



2.3. Subcloning the ColIb *psiB* gene.

To confirm that ColIb carries a *psiB* gene capable of conferring a Psi$^+$ phenotype on the host cell and to locate the gene more precisely, subclones of the region identified by Southern hybridization as containing the putative *psiB* gene were constructed and tested in GC4597 (*recA441 sfiA::lacZ*), JM12 λ^+ (*recA441*) and GY7221 (*recA730 sfiA::lacZ*) for the Psi$^+$ phenotype.

Although it appeared from the Southern hybridization data that the ColIb *psiB* gene is located on a 2.0 kb *Sal*I-*Pst*I fragment, initial subcloning experiments used larger fragments of ColIb. This was to avoid incurring problems due to the requirement of additional sequences for production of the Psi phenotype.

2.4. Construction of pALS4.

Plasmid pLG288, a derivative of ColIb*drd-1* containing a Tn*903* based Km^r insertion in the *ssb* gene, confers a Psi phenotype on the host cell whereas the parental ColIb*drd-1* plasmid does not (Table 2.1; Fig 2.4). The 11.4 kb *Sal*I S4 fragment of pLG288 was inserted into the *Sal*I site in the Tc^r gene of pBR328. This was achieved by ligation of *Sal*I digested pLG288 and pBR328 in a shotgun cloning experiment, using the Km^r determinant of the insert in *ssb* as a selectable marker for recombinants carrying the correct fragment. The resulting construct, pALS4, contains the region homologous to the R6-5 *psiB* probe and the ColIb *ssb* gene interupted by the Tn*903* derived kanamycin resistance cassette (Fig. 2.3). Previous attempts to isolate the corresponding S4 fragment from ColIb*drd-1*, which would differ from the pLG288 S4 fragment only in the lack of an insert in *ssb*, always resulted in derivatives which had suffered spontaneous deletions (Howland *et al.*, 1989, Howland 1989). Plasmid pALS4 showed no evidence of similar

Fig. 2.3. Restriction maps of pALS4, pALCS and pAL1.

Line 1 (top) shows restriction sites in the E1 *Eco*RI fragment of pLG288, the location of *oriT*, the Km^r determinant (hatched box) within the *ssb* gene and the putative location of *psiB*. Plasmids shown below are linearized at one end of their vectors (filled boxes; pBR328 for pALS4, pBluescript (SK) for pALCS and pAL1). The location and orientation of the *lacZ* promoter (plac) on the pBluescript vector is shown. Restriction sites are *Acc*I (A), *Cla*I (C), *Eco*RI (E), *Pst*I (P) and *Sal*I (S).

Relevant restriction enzyme fragment sizes of pALS4 were as follows:

Enzyme	Fragment sizes (kb)	Total (kb)
*Sal*I	11.4, 4.9	16.3
*Pst*I	8.2, 3.9, 3.0, 1.2	
*Sal*I/*Pst*I	5.2, 3.0, 2.9, 2.0, 2.0, 1.2	
*Cla*I	8.0, 6.0, 2.3,	
*Cla*I/*Sal*I	5.3, 4.3, 3.8, 2.3, 0.6	

Fig.2.3.

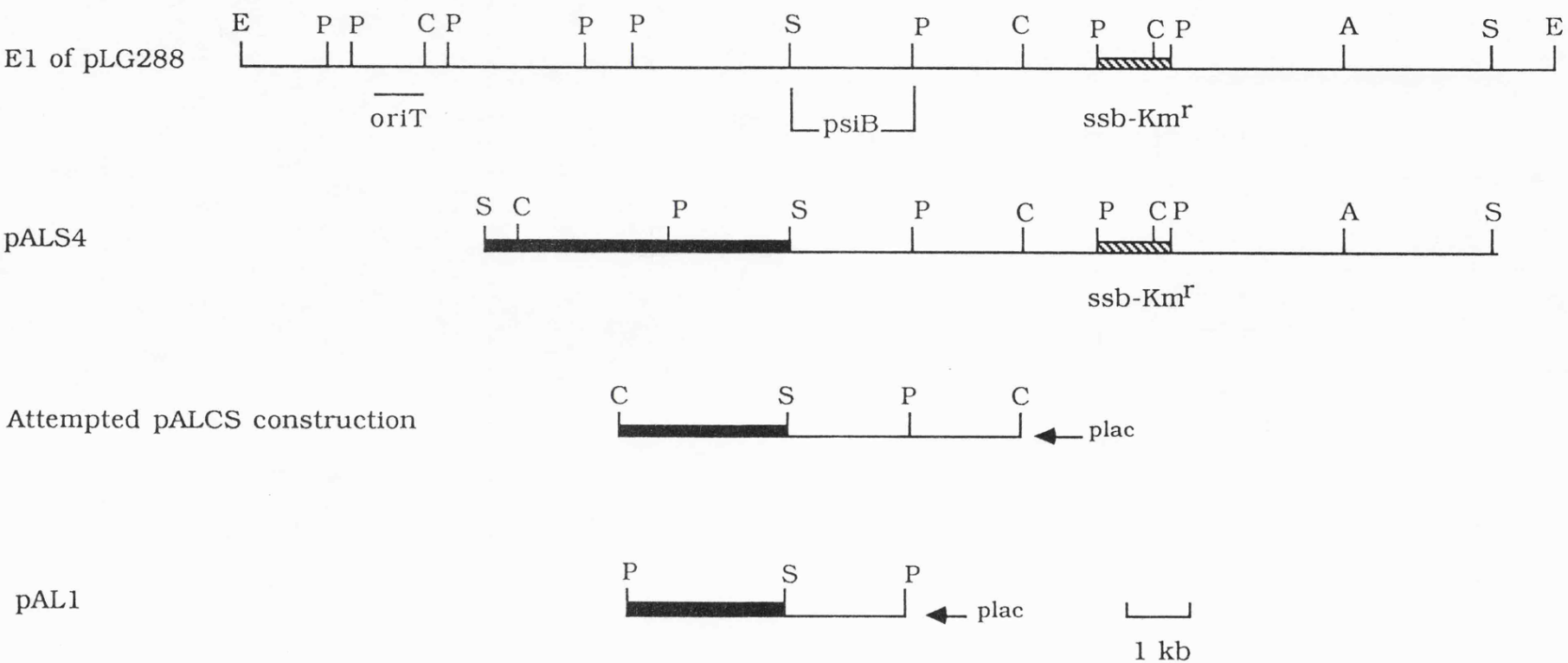

E1 of pLG288
E P P C P P P S P C P C P A S E
oriT
psiB
ssb-Km^r
pALS4
S C P S P C P C P A S
ssb-Km^r
Attempted pALCS construction
C S P C
plac
pAL1
P S P
plac
1 kb

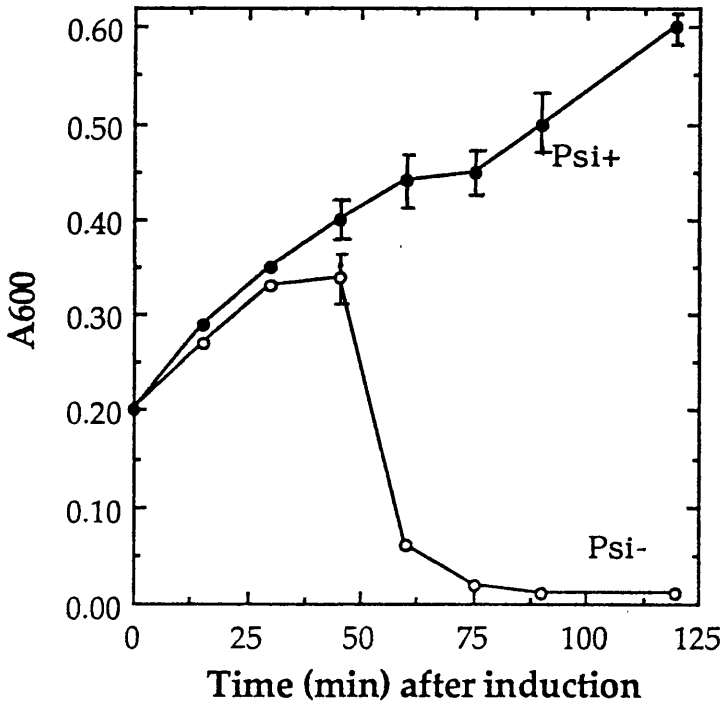
Table 2.1. Effect of plasmids on *sfiA::lacZ* expression in *recA441* and *recA730* cells.

	β-galactosidase in strain*		
Plasmid	GC4597 (30°C)	GC4597 (42°C)	GY7221
None	56	2320	2997
ColIb*drd-1*	45	1700	2780
pLG288	43	120	230
pALS4	61	145	285
pLG283	55	320	512
pAL1	NT	NT	3153
pAL1000	NT	NT	538
pAL1001	NT	NT	156
pBluescript (SK)	1938	3122	2884

* Values are units mg^{-1} protein. Each value is the mean of at least two experiments. NT, not tested.

Fig 2.4. λ induction in *recA441* cells.

Effect of plasmids on prophage λ induction in a *recA441* host. At time 0, cells growing at 30°C were shifted to 42°C, and adenine added to the medium to 0.5 mM. Strains containing pLG288, pALS4, pLG283, pAL1000 or pAL1001 showed no cell lysis and the plasmids were designated Psi$^+$ (closed circles). Strains containing no plasmid, ColIb*drd-1* or pAL1 lysed approximately 60 minutes after temperature shift and the plasmids were designated Psi$^-$ (open circles). Error bars show the upper and lower A_{600} values obtained.



deletions. This observation was not pursued further. Plasmid pLG283, constructed by C.Howland, was used for this work. This plasmid has a 2.35 kb deletion upstream of the *ssb* gene but was thought to be unaffected in the regions relevant to *psiB* (Fig. 2.1.).

Both pALS4 and pLG283 were tested in strains GY7221, GC4597 and JM12 λ^+ for the presence of the Psi phenotype (Table 2.1, Fig 2.4.). GY7221 has a *recA730* mutation and expresses SOS functions constitutively whereas GC4597 and JM12 λ^+ have the *recA441* mutation with SOS functions expressed at 42°C. Both plasmids were found to confer a Psi phenotype on the host cell. They prevented induction of the λ prophage and inhibited expression of *sfiA* under SOS inducing conditions. Plasmid pALS4 shows a slightly stronger Psi function than pLG283, measured by the *sfiA::lacZ* fusions, but the λ induction data shows no variations.

Plasmids pALS4 and pLG283 both contain large fragments of ColIb (11.2 kb and 7.75 kb respectively) and subsequent subcloning experiments using smaller fragments were required. An attempt was made to subclone a 3.8 kb *Cla*I-*Sal*I fragment from pALS4 (ColIb coordinates 20.2-24.0) into pBluescript(SK$^-$) utilising the *Cla*I and *Sal*I sites in the polylinker of the vector. This construct should contain the region showing homology to the R6-5 *psiB* probe but not the *ssb* gene. Figure 2.3 shows the attempted construction which was termed pALCS. The fragment was isolated from pALS4 and not pLG283 to avoid the possibility that the deletion suffered by pLG283 had also affected another part of the plasmid. Although white colonies were obtained indicating that the ligation had worked, the colonies were small and displayed an extremely slow growth rate. This subcloning experiment was repeated using different isolates of DNA and a variety of strains for the transformation, but the result was always the same. It appeared that the attempted construction was in some way detrimental to the cell. It is possible that the Bluescript vector has too high a copy number

and certain proteins are overexpressed which interfere with cell growth. Alternatively, regulatory elements may be lacking which previously controlled expression of a gene on this 3.8 kb *Cla*I/*Sal*I fragment.

2.5. Constuction of pAL1

The 2.0 kb *Sal*I-*Pst*I fragment identified in section 2.2. by Southern hybridization as containing the sequence homologous to R6-5 *psiB* was ligated to *Sal*I-*Pst*I cleaved pBluescript(SK) vector DNA (Fig. 2.3.). The 2.0 kb fragment was isolated from pALS4 and not pLG283 for the reasons outlined above. This work was undertaken at the same time as the subcloning of the 3.8kb *Cla*I-*Sal*I fragment described above, so the problems incurred with the use of the pBluescript vector had not yet been encountered. The SK orientation of vector was chosen so that transcription driven from the *lacZ* promoter of the vector would procede through the *Pst*I site first (Fig. 2.3.). An assumption was made that the ColIb *psiB* gene would be orientated in the same way as the F and R6-5 *psiB* genes, with transcription towards the *oriT* site. Hence this construct should be correctly orientated with the promoter located upstream of the *psiB* gene.

The construct, pAL1 was tested for the presence of a Psi$^+$ phenotype in strains GY7221 and JM12 λ^+ but not in GC4597. This is because the pBluescript vector contains part of the *lac* operator sequence which when present in multicopy, titrates out the chromosomally encoded *lac* repressor resulting in production of β-galactosidase from the chromosomal *lacZ* gene. Strain GY7221 has a deleted *lacZ* gene so this is not a problem. However, GC4597 has an intact *lacZ* gene and could not be used as the Psi test is based on assaying β-galactosidase specified by a *sfiA::lacZ* fusion. This problem is highlighted in Table 2.1. The presence of the pBluescript vector in GC4597 results in high levels of β-galactosidase even before SOS induction is triggered by the temperature shift.

From Table 2.1 and Fig 2.4. it can be seen that pAL1 fails to confer a Psi phenotype: presence of the plasmid did not prevent lysis of the *recA441* λ lysogen and expression of *sfiA::lacZ* was high in GY7221. As the original probe used to determine whether ColIb carries a *psiB* gene only contained ~80% of the R6-5 *psiB* gene, it appeared likely that pAL1 did not contain the entire ColIb *psiB* gene necessary for expession of the Psi phenotype.

A second Southern hybridization experiment was performed to determine more precisely the location of the sequence showing homology to the R6-5 *psiB* probe on pAL1. As a prerequisite to the Southern hybridization experiment, a more detailed restriction map of the 2.0 kb *Sal*I-*Pst*I fragment of pAL1 was generated (Fig. 2.5.). A wide selection of restriction enzymes were tested but, only *Hinc*II and *Bgl*I proved to be of use. The others tended to show clustering of sites towards one end of the fragment or cut the DNA too frequently.

Figure 2.6 shows that hybridization of the 0.5 kb *Eco*RV fragment of pGY7568 (carrying the R6-5 *psiB* probe) occurred to two *Hinc*II fragments of 3.4 kb and 1.5 kb. This result indicated that the putative ColIb *psiB* gene spans the *Hinc*II site at ColIb 22.4. The probe also hybridized to a 2.2 kb *Bgl*I fragment and to a 2.0 kb *Sal*I/*Pst*I fragment of pALS4, as expected. From these data, the approximate location of the ColIb *psiB* gene was determined to be towards the end of the fragment nearest the *Pst*I site used in construction of pAL1 (Fig. 2.5). The R6-5 *psiB* probe used lacks the first 60 bp of the gene but has 156 bp downstream. Assuming the direction of transcription of the R6-5 and ColIb *psiB* genes is the same (towards *oriT*, as for *ssb*) then it is possible that the *Pst*I site used in the construction of pAL1 is located within the ColIb *psiB* coding region. This was later confirmed using nucleotide sequence analysis and by introducing an insert at this *Pst*I site in a larger construct.

Fig. 2.5. Detailed restriction map of pAL1.

Line 1 (top) indicates kilobase coordinates on the ColIb map. Line 2 shows pAL1 linearized at one end of the pBluescript(SK) vector (stippled box). Relevant restriction sites are shown as is the location and orientation of the *lacZ* vector promoter (plac). Thick black lines correspond to restriction fragments that hybridize to the R6-5 *psiB* probe. Restriction sites are *Bgl*I (B), *Hinc*II (H), *Pst*I (P) and *Sal*I (S).

Restriction enzyme fragment sizes of pAL1 were as follows:

Enzyme	Fragment sizes (kb)	Total (kb)
*Pst*I	4.9	4.9 kb
*Sal*I	4.9	
*Sal*I/*Pst*I	2.9, 2.0	
*Hinc*II	3.4, 1.5	
*Bgl*I	2.2, 1.3, 1.1, 0.3	
*Hinc*II/*Pst*I	2.9, 1.5, 0.5	
*Bgl*I/*Pst*I	1.35, 1.3, 1.1, 0.85, 0.3,	
*Bgl*I/*Sal*I	2.2, 1.3, 0.85, 0.3, 0.3	4.95 kb

Fig.2.5.

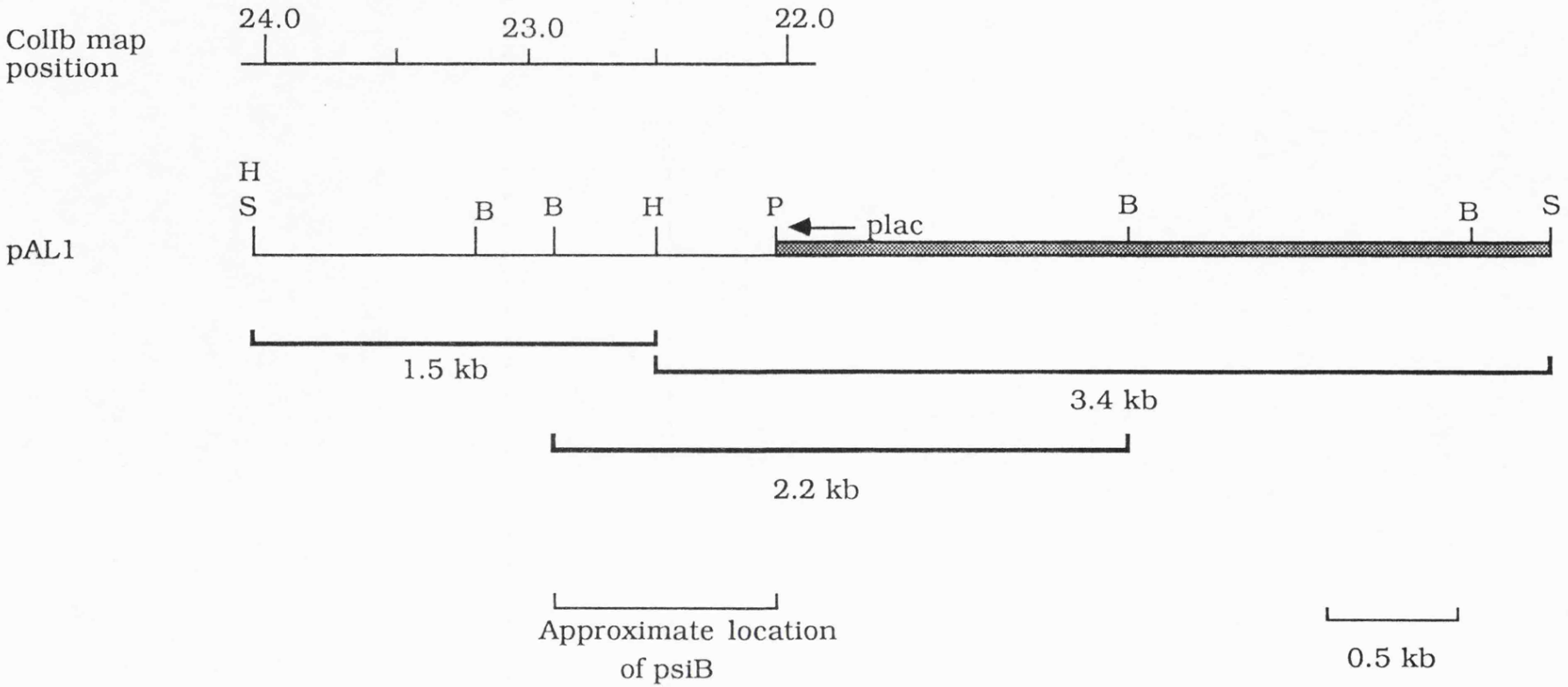
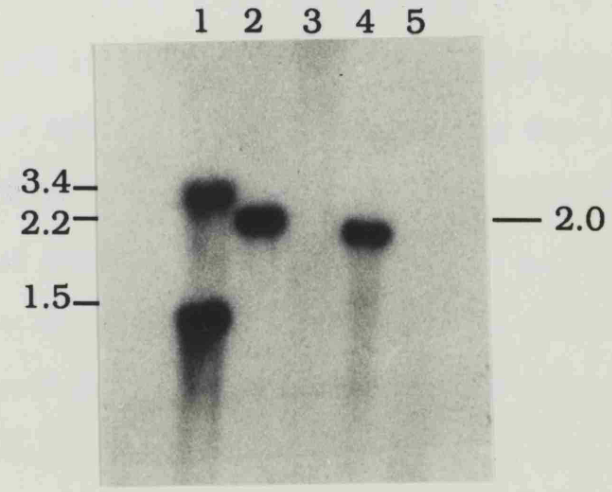

ColIb map position
24.0
23.0
22.0
pAL1
H
S
B
B
H
P
plac
B
B
S
1.5 kb
3.4 kb
2.2 kb
Approximate location of psiB
0.5 kb

Fig. 2.6. Results of a Southern hybridization of a 0.5 kb *Eco*RV fragment carrying R6-5 *psiB* to restriction fragments of pAL1.

Lanes correspond to (1) pAL1 x *Hinc*II, (2) pAL1 x *Bgl*I, (3) *E. coli* chromosomal DNA x *Sal*I, (4) pALS4 x *Sal*I/*Pst*I and (5) *E. coli* chromosomal DNA x *Eco*RI, separated by agarose gel electrophoresis.



The Southern hybridization data shown in Fig. 2.6 had also included an *EcoR*I and *Sal*I digest of *E. coli* chromosomal DNA (kindly provided by Serge Casegrola). At the final stringency used (1 x SSC, 0.1 % SDS, 65°C) there was no hybridization observed.

2.6. Construction of pAL1000 and pAL1001

Following the problems encountered with the use of the Bluescript vector, a vector with a lower copy number was chosen for the construction of plasmids pAL1000 and pAL1001. The vector chosen was pHG165, which is a pUC based vector with a pBR copy number due to the introduction of the *rop* gene from ColE1 (Stewart *et al.*, 1986). A 2.7 kb *Sau3*A fragment from pALS4 had been identified during a search for suitable restriction sites for subcloning the *psiB* gene (data not shown). This fragment, which has the putative *psiB* coding region located towards one end, was inserted into the *BamH*I site of the polylinker in pHG165 in both orientations to produce pAL1000 and pAL1001 (Fig. 2.7.). An attempt was also made to subclone this 2.7 kb *Sau3*A fragment into the pBluescript vector, but as for the 3.8 kb *Cla*I-*Sal*I fragment (see section 2.4.), only small colonies were obtained which displayed very slow growth. Strains containing pAL1000 and pAL1001 did not show any growth defects and thus it would appear that the use of the lower copy number vector, pHG165, overcomes the problem. Both pAL1000 and pAL1001 display a Psi$^+$ phenotype as tested in strains GY7221 and JM12 λ^+ (Fig. 2.4, Table 2.1). Unfortunately pHG165, like pBluescript carries part of the *lac* operator sequence and so the constructs could not be tested in GC4597. Plasmid pAL1001 conferred a slightly stronger Psi phenotype than pAL1000. This may reflect the orientation of the *psiB* gene relative to the *lac* promoter of the vector (Fig. 2.7.). If the *psiB* gene of ColIb is transcribed in the same dirction as that of F, then in pAL1001 *psiB* is located downstream of the promoter of the vector in the correct transcriptional orientation. The

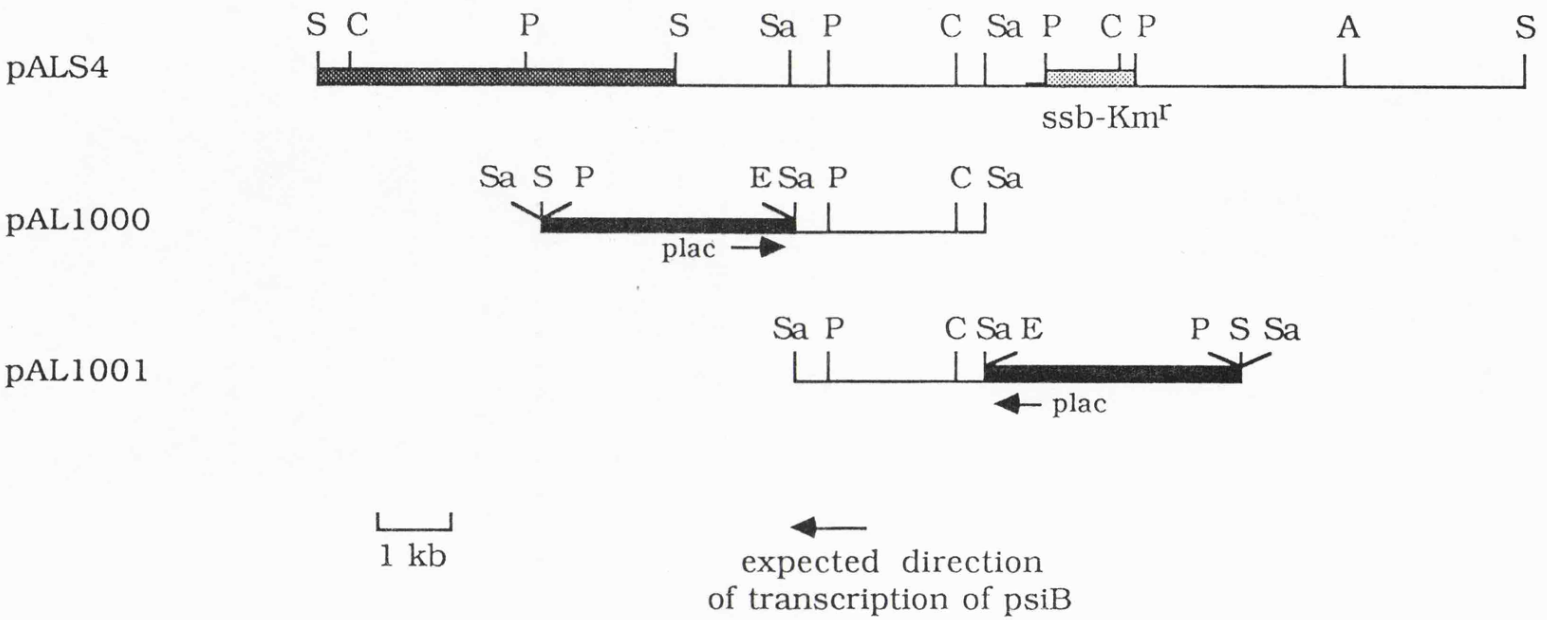
Fig. 2.7. Restriction maps of plasmids pAL1000 and pAL1001.

Line 1 (top) shows a restriction map of pALS4 including the Km^r determinant within *ssb*. Lines 2 and 3 show pAL1000 and pAL1001 linearized at one end of the vector portion, the position and orientation of the *lacZ* promoter (plac) of the vector is indicated. Vectors are pBR328 for pALS4 (stippled box) and pHG165 for pAL1000 and pAL1001 (filled box). Restriction sites are *Acc*I (A), *Cla*I (C), *Eco*RI (E), *Pst*I (P), *Sal*I (S) and *Sau*3A (Sa). Only the two relevant *Sau*3A sites are shown.

Restriction enzyme fragment sizes were as follows:

	Enzyme	Fragment sizes	Total
a) pAL1000	*Eco*RI	6.0	6.0 kb
	*Sal*I	6.0	
	*Pst*I	3.75, 2.25	
	*Pst*I/*Eco*RI	3.3 ,2.25, 0.44	
	*Pst*I/*Sal*I	3.75, 2.25	
b) pAL1001	*Eco*RI	6.0	
	*Sal*I	6.0	
	*Pst*I	5.55, 0.44	
	*Pst*I/*Eco*RI	3.3, 2.25 ,0.44	
	*Pst*I/*Sal*I	5.55, 0.44	

Fig.2.7.


pALS4
S C P S Sa P C Sa P C P A S
ssb-Km^r
pAL1000
Sa S P E Sa P C Sa
plac
pAL1001
Sa P C Sa E P S Sa
plac
1 kb
expected direction
of transcription of psiB

fact that both pAL1000 and pAL1001 display a Psi$^+$ phenotype indicates that there is a promoter located upstream of *psiB* on the 2.7 kb *Sau*3A fragment.

2.7. Determination of the nucleotide sequence of the ColIb *psiB* gene

The work described above indicates that the ColIb *psiB* gene is located close to the *Pst*I site at ColIb coordinates 22.1 and that the *Pst*I site may in fact lie within the *psiB* coding region. Thus it was decided to determine the nucleotide sequence in the region proximal to this *Pst*I site in order to show whether the site is located within the PsiB coding region. The nucleotide sequence would also indicate the extent of identity between a *psiB* gene from an I1 plasmid (ColIb) and *psiB* genes from F-like plasmids (F and R6-5), for which sequence data was available (Dutreix *et al.*, 1988).

The 2.0 kb *Sal*I-*Pst*I fragment from pAL1 was inserted into M13mp19. As the *Sal*I and *Pst*I sites in the M13mp19 polylinker are located adjacent to each other and therefore may not be cleaved in a double *Sal*I/*Pst*I digest, the 2.0 kb fragment was excised from pAL1 utilising the *BamH*I site of the Bluescript polylinker instead of the *Pst*I site and inserted into M13mp19 cleaved with *BamH*I and *Sal*I (Fig 2.8.).

Recombinant M13 single stranded DNA was prepared and 334 bp sequenced using the universal primer. The sequence data obtained showed extensive identity to the F and R6-5 *psiB* genes, although the sequence obtained did not contain the entire gene. There was a region towards the primer distal end of this sequence that diverges quite considerably from the corresponding region of F and R6-5. As this region was at the upper limit of resolvation of the sequencing gel, three further primers were designed to enable sequence data of the entire ColIb *psiB* gene to be determined and the original sequence be verified by sequencing the complementary strand where ambiguities arose. Double stranded plasmid sequencing of pALS4 was used to complete the sequence using the B, C and D primers (Fig. 2.10).

Figure 2.8. Sequencing strategy.

Line 1 (top) shows pAL1 linearized at a site in the pBluescript vector. A 2.0 kb *Sal*I/*Bam*HI fragment from pAL1 was inserted into *Sal*I/*Bam*HI cleaved M13mp19. The approximate location of the sequencing primers is shown (A; M13 universal primer and B,C and D; primers complementary to *psiB*). Sequence was initially obtained from single-stranded recombinant M13 DNA using primer A. Primers B, C and D were used to obtain sequence from denatured duplex pALS4 DNA. Restriction sites are *Bam*HI (B), *Hinc*II (H), *Pst*I (P) and *Sal*I (S).

Fig.2.8.

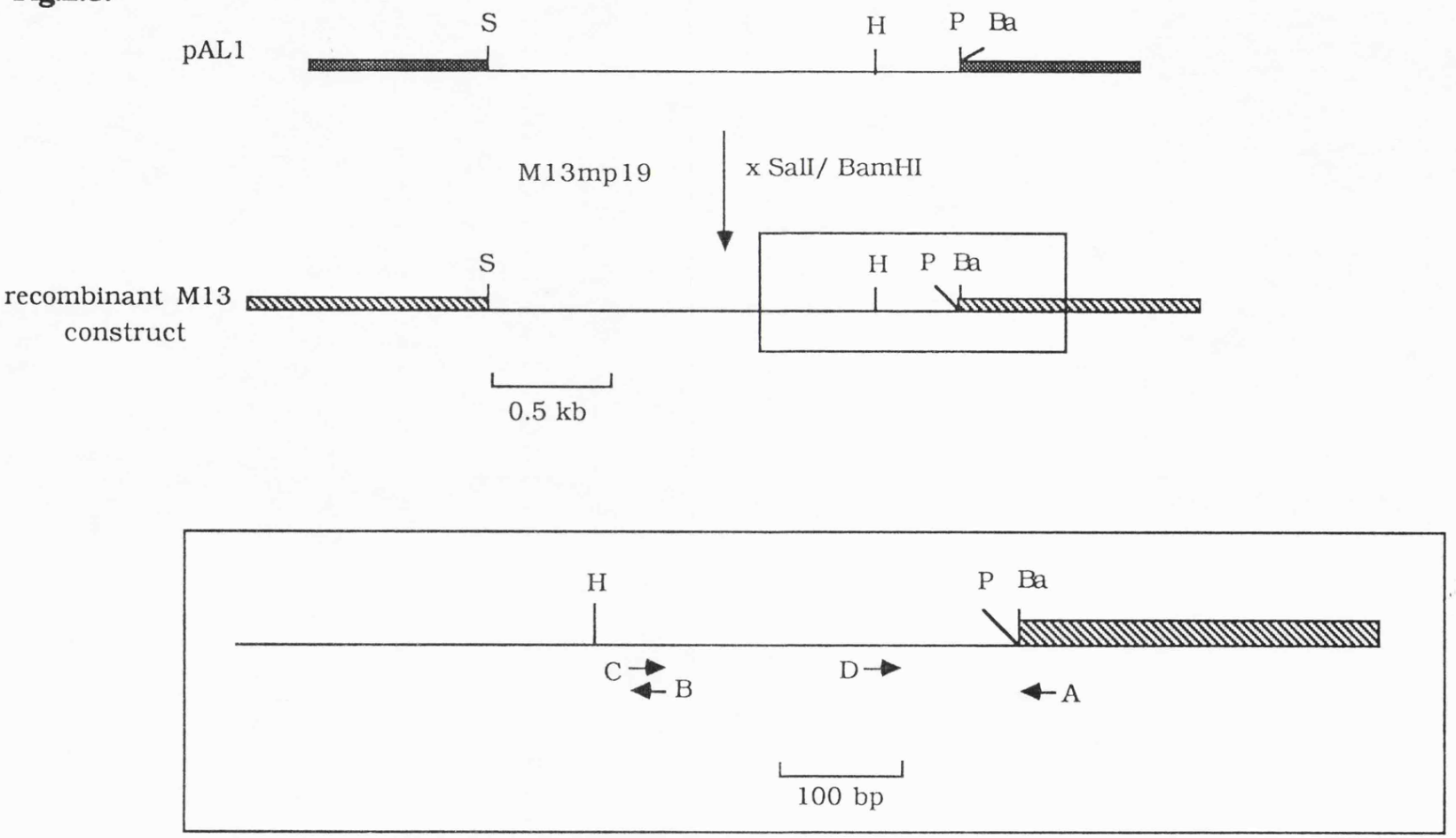

pAL1
S
H
P
Ba
M13mp19
x SalI/ BamHI
recombinant M13
construct
S
H
P
Ba
0.5 kb
H
P
Ba
C
B
D
A
100 bp

Fig. 2.9. Representative sequencing gel.

Tracks are (1) M13 recombinant sequenced using the universal primer and (2) plasmid pALS4 sequenced using primer B. The tracks labelled C, A, T and G refer to reactions terminated with ddCTG, ddATG, ddTTG and ddGTG, respectively.

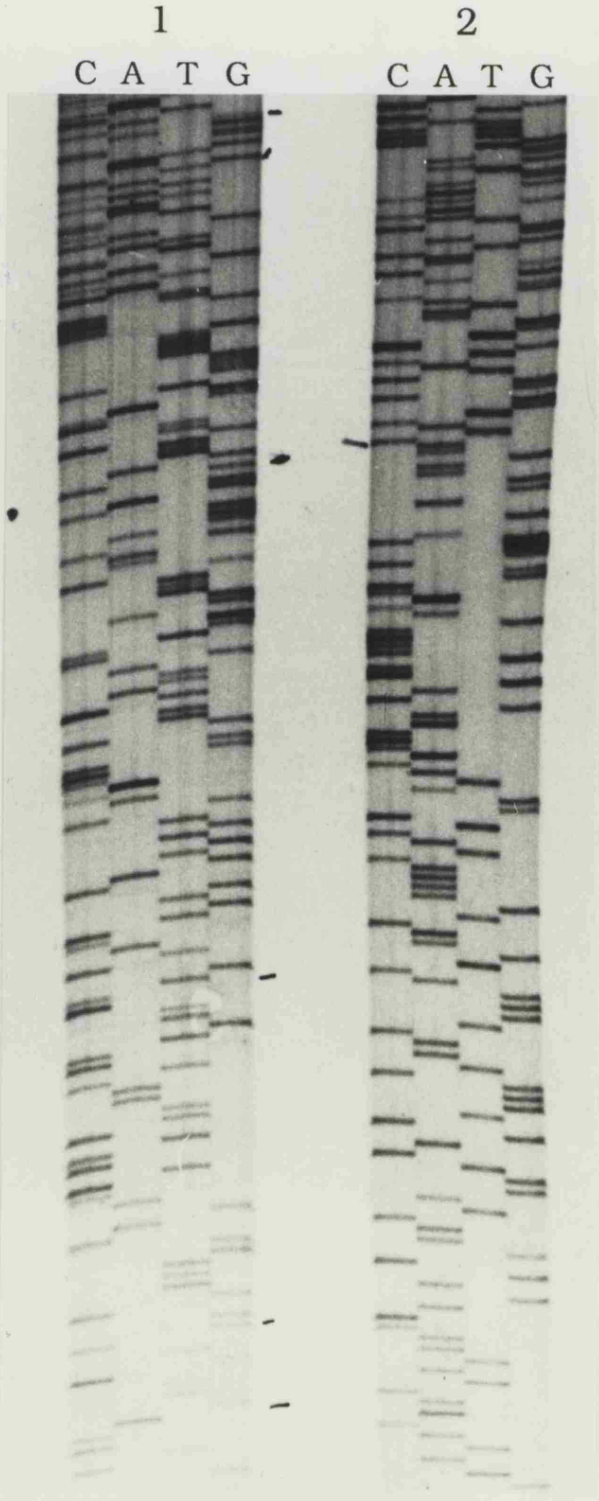
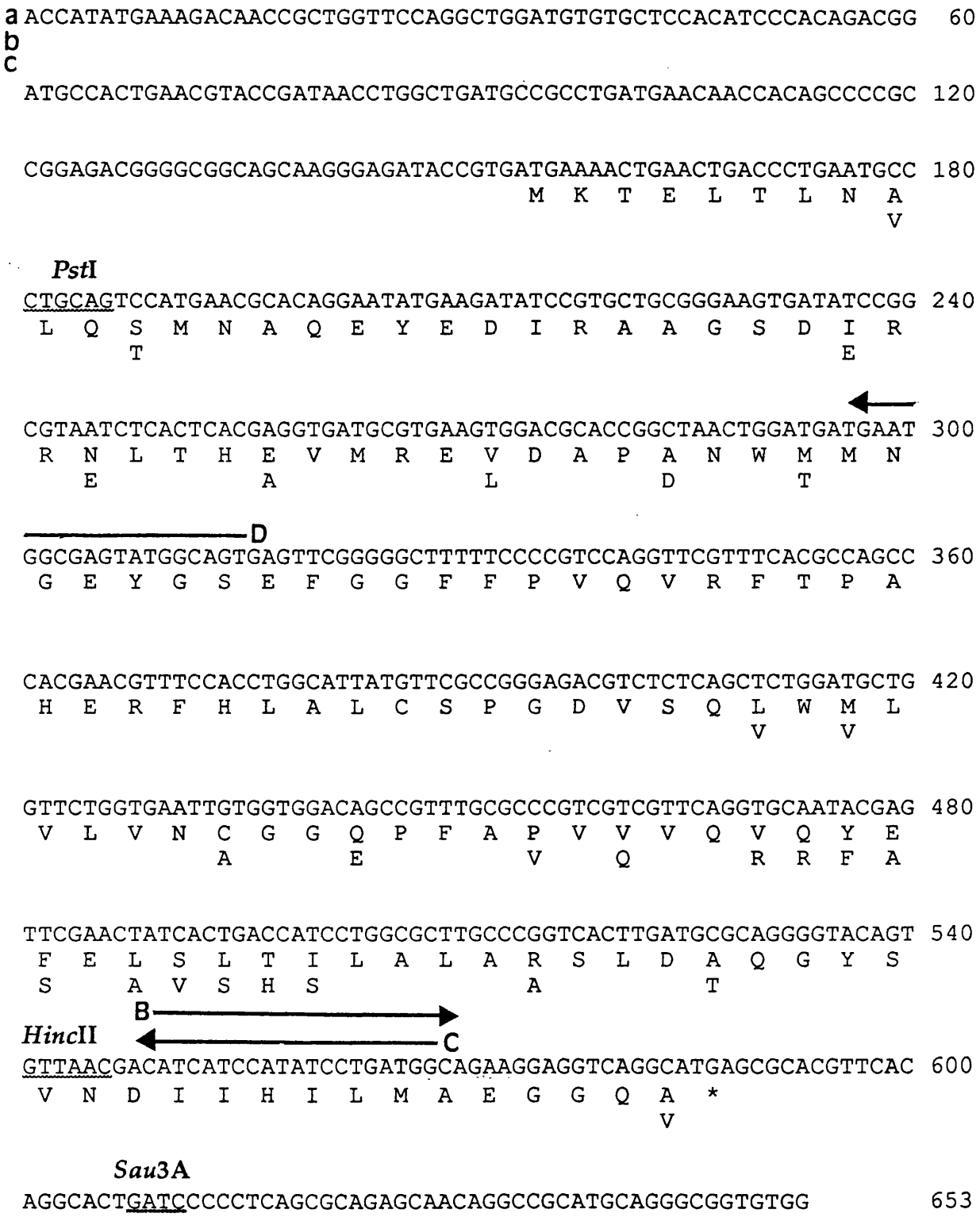


Fig. 2.10.

(a) Nucleotide and (b) predicted amino acid sequence of the ColIb *psiB* gene and protein. The amino acid sequence of F PsiB (c) is shown only where differences from ColIb PsiB occur. It has been assumed that the initiation and termination codons are identical to those of F *psiB* (Dutreix *et al.*, 1988). 159 bp upstream and 69 bp downstream of the ColIb *psiB* coding region are shown. *Pst*I, *Hinc*II and *Sau*3A restriction sites are indicated. The *Pst*I site was used in construction of pAL1 and the *Sau*3A site in the construction of pAL1000 and pAL1001. Section 2.5 described an experiment which indicated that the ColIb *psiB* gene spanned a *Hinc*II site. Sequences corresponding to the primers B, C and D, and the direction of extension, are represented by arrows.


a
b
c
ACCATATGAAAGACAACCGCTGGTTCCAGGCTGGATGTGTGCTCCACATCCCACAGACGG 60
ATGCCACTGAACGTACCGATAACCTGGCTGATGCCGCCTGATGAACAACCACAGCCCCGC 120
CGGAGACGGGGCGGCAGCAAGGGAGATACCGTGATGAAAACTGAACTGACCCTGAATGCC 180
M K T E L T L N A
V
PstI
CTGCAGTCCATGAACGCACAGGAATATGAAGATATCCGTGCTGCGGGAAGTGATATCCGG 240
L Q S M N A Q E Y E D I R A A G S D I R
T E
CGTAATCTCACTCACGAGGTGATGCGTGAAGTGGACGCACCGGCTAACTGGATGATGAAT 300
R N L T H E V M R E V D A P A N W M M N
E A L D T
D
GGCGAGTATGGCAGTGAGTTCGGGGGCTTTTTCCCCGTCCAGGTTCGTTTCACGCCAGCC 360
G E Y G S E F G G F F P V Q V R F T P A
CACGAACGTTTCCACCTGGCATTATGTTCGCCGGGAGACGTCTCTCAGCTCTGGATGCTG 420
H E R F H L A L C S P G D V S Q L W M L
V V
GTTCTGGTGAATTGTGGTGGACAGCCGTTTGCGCCCGTCGTCGTTCAGGTGCAATACGAG 480
V L V N C G G Q P F A P V V V Q V Q Y E
A E V Q R R F A
TTCGAACTATCACTGACCATCCTGGCGCTTGCCCGGTCACTTGATGCGCAGGGGTACAGT 540
F E L S L T I L A L A R S L D A Q G Y S
S A V S H S A T
B
HincII
C
GTTAACGACATCATCCATATCCTGATGGCAGAAGGAGGTCAGGCATGAGCGCACGTTCAC 600
V N D I I H I L M A E G G Q A *
V
Sau3A
AGGCACTGATCCCCCTCAGCGCAGAGCAACAGGCCGCATGCAGGGCGGTGTGG 653

653 bp were sequenced, which include the putative *psiB* coding region and 159 bp upstream and 69 bp downstream of the gene. The complete sequence, indicating the location of the primers and relevant restriction enzyme sites is shown in Fig. 2.10. Also shown is the predicted amino acid sequence of the ColIb PsiB polypeptide and any differences that exist with F PsiB. Figure 2.9 shows part of a representative sequencing gel.

Some regions were not sequenced on both strands, but these regions did show total or almost total identity with the F or R6-5 sequence. It has been assumed that the ColIb and F *psiB* genes share the same translational initiation and termination codons, although I have no supporting evidence other than the high degree of similarity between both plasmids in this region.

There is a *Pst*I site located 29 bp into the coding region of *psiB*. This site maps in approximately the same position as the *Pst*I site used in the construction of pAL1. The Southern hybridization experiment described in section 2.5. indicated that the ColIb *psiB* gene spans a *Hinc*II site at ColIb map position 22.4. From analysis of the sequence data there is a *Hinc*II site located within the *psiB* gene towards the 3′ end (Fig 2.10.). The direction of transcription of ColIb *psiB* is the same as that of F, with transcription proceding towards the *oriT* site. The *ssb* gene of both plasmids is also transcribed in this direction.

2.8. Discussion

Presence of a *psiB* gene on ColIb was first indicated by the observation that an insertion in the plasmid *ssb* gene resulted in the production of a Psi^+ phenotype (Howland *et al.*, 1989). In this chapter the location and nucleotide sequence of the ColIb *psiB* gene is described. Using part of the R6-5 *psiB* gene as a probe in Southern hybridization experiments, a homologous sequence was located in the leading region of ColIb. The gene

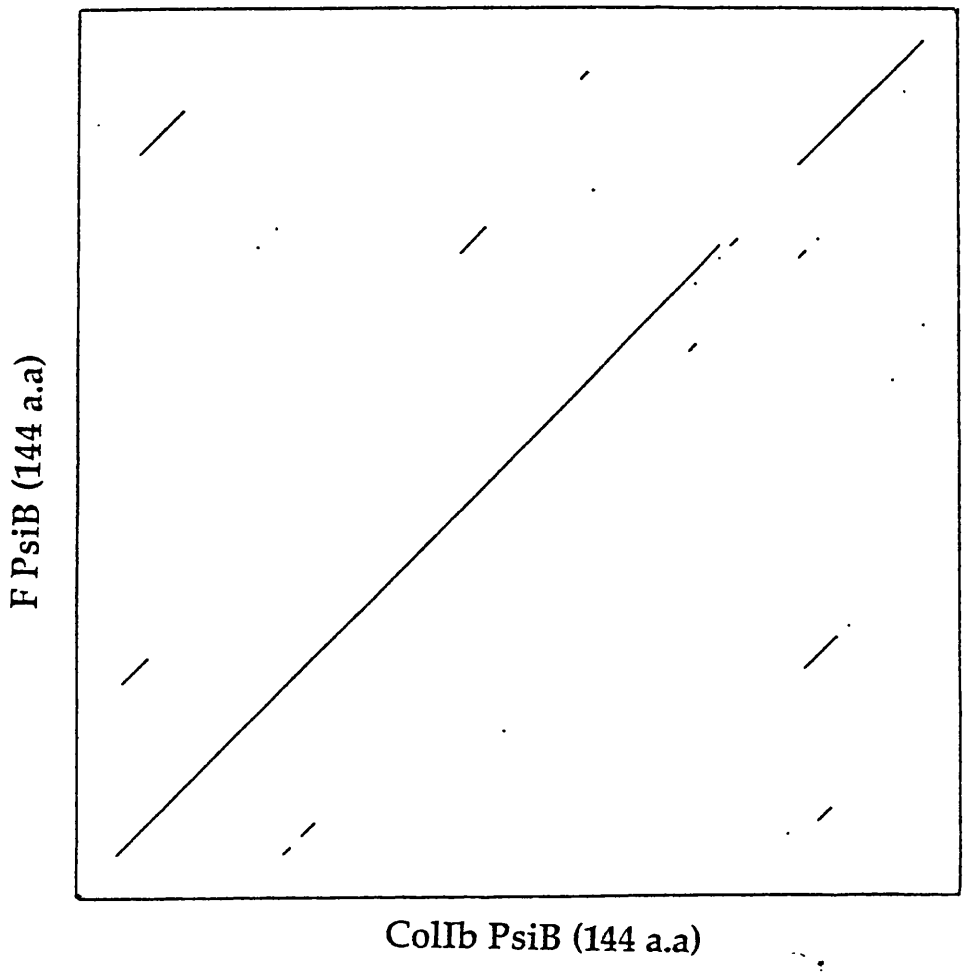
was more precisely located using subcloning and nucleotide sequence analysis.

There is 84% identity at the nucleotide level between the *psiB* genes of F and ColIb, which is approximately the level of identity between the respective plasmid *ssb* genes. Moreover, the genes map in the same relative position in the leading region of the plasmids downstream of an *ssb* gene. Conservation of this segment of F and ColIb is underscored by the similar size (2.5 kb) of the region between *psiB* and *ssb* on the two plasmids. The high degree of conservation may imply that this region has an important role in plasmid biology and in particular for conjugation, since all plasmids that carry *psiB* and *ssb* genes are self-transmissable but specify apparently distinct conjugation systems. The nucleotide sequence analysis showed that the region of identity also extends 159 bp upstream and 69 bp downstream of ColIb *psiB* gene, which delimit the sequence data. These regions show 83% and 88% identity respectively with the corresponding portions of plasmid F (Dutreix *et al.*, 1988). The *psiA* gene of F is located immediately downstream of *psiB* (Loh et al., 1990) and so it appears that ColIb might also encode a *psiA* gene. The function of *psiA* is unknown, since *psiB* alone is sufficient for the Psi function (Bailone *et al.*, 1988).

Although the *psiB* genes of ColIb and F/R6-5 are very similar and hence have similar predicted amino acid compositions, the similarity is greatest in the amino terminal region. Dotplot comparisons of amino acid sequence illustrate this point well, showing that there is a region towards the carboxy terminus of the polypeptide where the sequence has diverged (Fig. 2.11). A similar observation is not found when the F and R6-5 PsiB proteins are compared, these proteins share 96% identity. The significance of this region of divergence is unclear as it is localised. It may reflect a region required for specific interaction with other plasmid encoded products or simply a region where mutations can accumulate with no detrimental

Fig. 2.11. Dot plot amino acid comparison of ColIb PsiB and F PsiB.

Parameters used were a window of 12 and a stringency of 8.



effects. The carboxy terminus of the R6-5 PsiB protein can be deleted without loss of Psi function indicating that this region is outside the active centre of the protein (Bailone *et al.*, 1988). The apparent molecular weight of F and R6-5 PsiB proteins on SDS-PAGE is about 11-12 kD whereas that calculated from the sequence data is 15.7 kD (Dutreix *et al.*, 1988). Dutreix *et al.* (1988) found no evidence of postranslational modification of the PsiB proteins such as proteolytic processing. The predicted molecular weight of ColIb PsiB from the sequence data is 15.9 kD.

Psi activity was only observed in established strains when the intact ColIb*drd-1 psiB* gene is carried on a multicopy plasmid as for pALS4, pLG283, pAL1000 and pAL1001 or when ColIb*drd-1* carries an insert in *ssb* as in pLG288. Plasmid pALS4, which has an insert in the *ssb* gene, displayed a stronger Psi function than pLG283, which has a deletion upstream of the *ssb* gene (Fig. 2.1.) Although it is possible that the deletion suffered by pLG283 has altered the regulation of the *psiB* gene, I show in chapter 4 through the use of a promoter probe that the insert in the *ssb* gene of pLG288 results in increased transcription of the *psiB* gene. This observation provides an explanation for the greater Psi activity exhibited by pALS4 compared to pLG283, as pALS4 (derived from pLG288) carries such an insert in *ssb*, whereas pLG283 (derived from ColIb*drd-1*) does not. Plasmid pAL1, which carries the 2.0 kb *Sal*I-*Pst*I fragment showing homology to the R6-5 *psiB* probe failed to display a Psi$^+$ phenotype. The *Pst*I site used in the construction of pAL1 was shown from the nucleotide sequence to be located 29 bp into the PsiB coding region. The introduction of a 4.7 kb promoter probe at this *Pst*I site in pAL1001 inactivates the Psi function. This aspect of the work is described in Chapter 3 in detail. Both pAL1000 and pAL1001 which differ only in the orientation of the ColIb fragment in the polylinker portion of the vector, confer strong Psi$^+$ phenotypes. This suggests that there is a promoter located downstream of *ssb* and that it is possible to

express *psiB* without expression of *ssb*. pAL1001 confers a stronger Psi$^+$ phenotype than does pAL1000, presumably reflecting the orientation of the *psiB* gene relative to the *lacZ* promoter of the vector. In pAL1001, *psiB* should be correctly orientated with respect to the vector promoter if the gene is transcribed in the same orientation as that of F and R6-5.

It is not clear from this work if SOS inhibition is the primary physiological role of *psiB*. It was proposed by Bagdasarian *et al.*, 1986, that the Psi function has evolved on conjugative plasmids to allow transfer of single-stranded DNA without triggering the SOS response of the host cell. The experiments described here and elsewhere (Bagdasarian *et al.*, 1986; 1988; Bailone *et al.*, 1988) made use of RecA mutants which are easily triggered for SOS functions. Indeed, Bailone *et al.* (1988) reported that for inhibition of the SOS response triggered by UV-irradiation of a RecA$^+$ host, the *psiB* gene must be present in high copy and under the control of an inducible promoter to facilitate SOS inhibition. This suggests that the *psiB* gene inhibits SOS induction as a function of the intracellular concentration of its product. Thus, if increased expression of *psiB* is required for the Psi phenotype, this raises the question of *psiB* function. No information was gained from the sequence data as to the mechanism of PsiB action. The predicted ColIb PsiB amino acid sequence does not show any similarity to others in the data bases.

Chapter 3.

Construction of *lacZ* transcriptional fusions to ColIb *ssb*, *psiB* and *sog* genes.

3.1. Introduction

In order to gain further information on the role of the genes located in the leading region, it was appropriate to investigate their mode of regulation. Studies on the ability of the *ssb* genes of ColIb and F-like plasmids to suppress *E. coli ssb-1* mutations indicated that the plasmid *ssb* genes were coordinately regulated with the transfer genes (Golub and Low, 1986a; Howland *et al.*, 1989). Furthermore, the SOS inhibitory phenotype was only observed when expression of *psiB* was altered (Bailone *et al.*, 1988; Dutreix *et al.*, 1988).

Initially, it was decided to measure *ssb* and *psiB* mRNA levels from ColIb and ColIb*drd-1*, to determine whether the genes are coordinately regulated with the ColIb transfer system at the transcriptional level. The nature of the *drd-1* mutation, which allows constitutive expression of the *tra* genes, is undefined. However, if the *ssb* and *psiB* genes were under the same type of control as the core *tra* genes, then higher levels of mRNA would be expected in the strains containing the ColIb*drd-1* plasmid.

The *sog* gene, which encodes a DNA primase active in conjugation, was chosen as a positive control for these regulatory studies. There is considerable genetic evidence to show that the *sog* gene is coordinately regulated as part of the ColIb transfer system and it is located within the core transfer region (Rees *et al.*, 1987). Such a positive control would enable the level and pattern of expression of leading region genes to be compared to that of a representative transfer gene.

Attempts to detect transcripts corresponding to *psiB* and *ssb* by northern hybridization and RNA dotblot analysis failed, although the RNA

extracted was deemed to be of good quality. It was concluded that the level of transcription of these genes may be too low for this approach to be productive. Indeed previous reports indicated that the level of transcription in the leading region of plasmid F is low (Cram *et al.*, 1984). I therefore decided to use an alternative method for analysing the regulation of expression of *ssb*, *psiB* and *sog*. The method chosen involved the creation of transcriptional fusions between these genes on ColIb and ColIb*drd-1* and *lacZ*, the β-galactosidase structural gene. Thus the transcription of the genes could conveniently be monitored by measuring β-galactosidase levels specified by the fusions. It was thought that the creation of such fusions would allow the transcription of the genes to be studied under a wider variety of conditions than would be possible with the RNA analysis and the technique would prove to be more sensitive.

This chapter concerns the construction of *lacZ* transcriptional fusions to cloned *ssb*, *psiB* and *sog* genes and the subsequent recombination of the fusions into ColIb and ColIb*drd-1*. A 4.7 kb fragment containing a promoterless *lacZ* gene and the kanamycin resistance determinant of Tn*903* was used in the construction of the transcriptional fusions (Kokotek and Lotz, 1989). The *lacZ* genes and Km^r genes on this fragment are transcribed towards each other and are separated by the bidirectional transcriptional terminator from phage fd (Fig. 3.1). The *lacZ* gene lacks a promoter but retains its Shine-Dalgarno sequence allowing creation of operon fusions. The kanamycin resistance gene is constitutively expressed, providing a selectable marker for the gene replacement strategy. Thus, the 4.7 kb *lacZ*-Km^r cassette can be introduced into a target gene and transcription of the DNA mutagenised by the insert can be monitored by assaying β-galactosidase activity.

3.2. Attempt to analyse *ssb* and *psiB* mRNA levels.

Total cellular RNA was extracted from BW85 (plasmid free), BW85 (ColIb) and BW85 (ColIb*drd-1*) using the method of Miller (1984). The integrity of RNA was analysed by running on formaldehyde gels, staining with ethidium bromide and looking for the presence of the two bands corresponding to the 23S and 16S ribosomal RNA species.

Attempts to detect transcripts corresponding to *ssb* or *psiB* mRNA, involved both northern hybridization and RNA slot blots. The probes used in these experiments were a 1.0 kb *Cla*I/*Pst*I fragment from pLG284 for *ssb* and a 0.44 kb *Hinc*II/*Pst*I fragment from pAL1 for *psiB*. No specific transcripts were detectable using these probes by either method and where hybridization occured, it was deemed to be none specific. Due to time constraints, this work was pursued no further.

3.3. Construction of a *lacZ* transcriptional fusion to the *ssb* gene of pLG284.

Plasmid pLG284 carries the *ssb* gene of ColIb on a 3.95 kb *Cla*I/*Sal*I fragment in a pACYC184-based vector (Fig. 3.2). This plasmid can be linearized at a single *Pst*I site located 94 bp into the *ssb* coding sequence (Howland *et al.*, 1989). The 4.7 kb *lacZ*-Km^r cassette has flanking *Pst*I sites and therefore was introduced into the *ssb* gene at this unique *Pst*I site (Fig. 3.2). Recombinant plasmids containing the insert were identified using the Km^r marker and analysed for the correct size of insert (4.7 kb = *lacZ*-Km^r) and vector (7.35 kb = pLG284). The *lacZ*-Km^r cassette has asymmetric *Eco*RI sites to facilitate orientation of the insert. Recombinant plasmids were obtained with the insert in both possible orientations. Plasmids in which the *lacZ* gene was in the same transcriptional orientation relative to that of the *ssb* gene were designated as having the ON orientation and those with the fragment in the opposite orientation as being NO. The same nomenclature is used for the inserts generated in the *psiB* and *sog* genes.

Fig. 3.1. The *lacZ*-Km^r promoter probe.

The location and direction of transcription of the Km^r determinant and the promoterless *lacZ* gene is shown. Restriction sites are *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P) and *Sal*I (S).

Fig. 3.2. Introduction of the *lacZ*-Km^r cassette into the *ssb* gene of pLG284.

Line 1 (top) shows the location and direction of transcription of the *ssb* gene on pLG284. Lines 2 and 3 show pAL4 and pAL5, which have the *lacZ*-Km^r cassette introduced at a *Pst*I site within the *ssb* gene in the ON and NO orientation, respectively. Restriction sites are *Cla*I (C), *Eco*RI (E), *Pst*I (P) and *Sal*I (S).

Restriction fragments are as follows:

	Enzyme	Fragment sizes (kb)	Total (kb)
a) pLG284	*Pst*I	7.35	7.35
	*Sal*I	7.35	
	*Eco*RI	7.35	
b) pAL4	*Pst*I	7.35, 4.7	12.0
	*Sal*I	12.0	
	*Eco*RI	4.6, 4.4, 3.0	
c) pAL5	*Pst*I	7.35, 4.7	12.0
	*Sal*I	12.0	
	*Eco*RI	6.3, 3.0, 2.7	

Fig.3.1

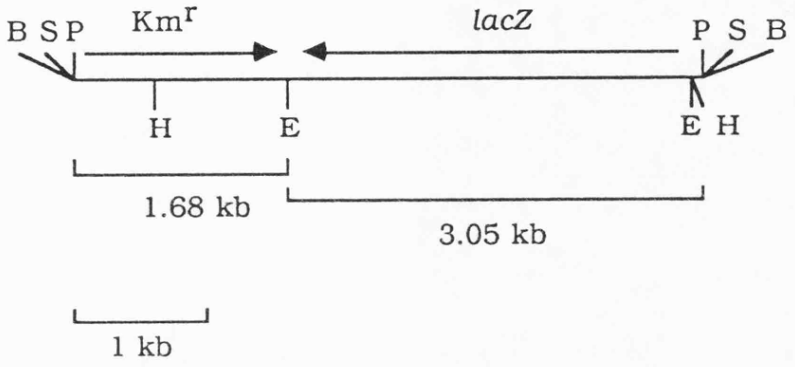
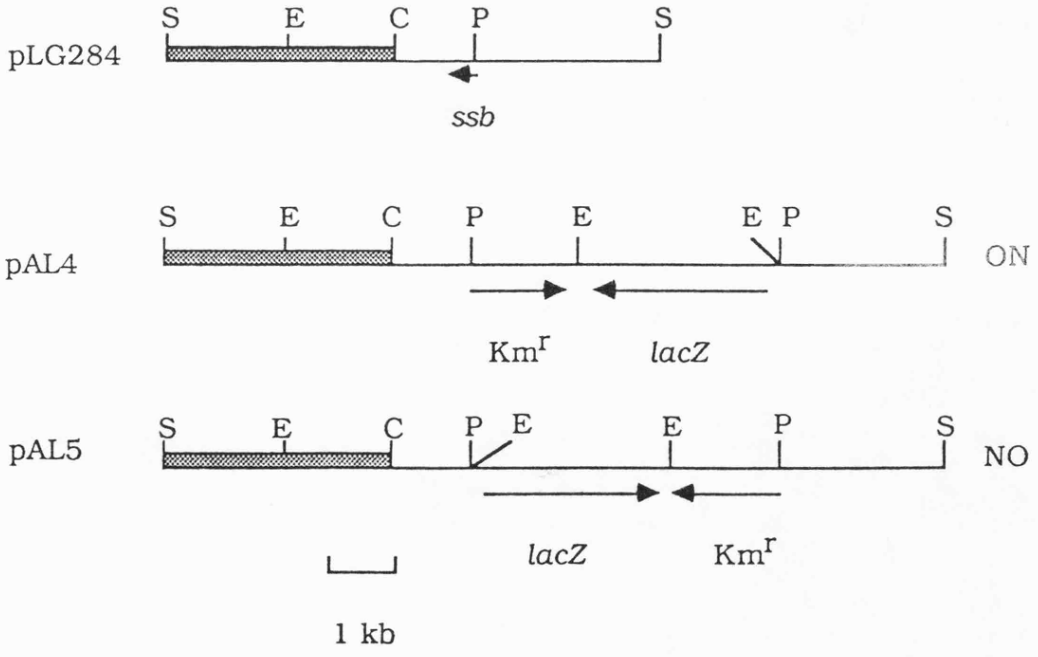

B S P
Km^r
lacZ
P S B
H
E
E H
1.68 kb
3.05 kb
1 kb

Fig.3.2.


pLG284
S E C P S
ssb
pAL4
S E C P E E P S
ON
Km^r
lacZ
pAL5
S E C P E E P S
NO
lacZ
Km^r
1 kb

Plasmids pAL4 and pAL5 are representatives of the recombinants with the *lacZ*-Km^r cassette in the ON and NO orientations respectively (Fig. 3.2).

To confirm that the insert had disrupted the plasmid *ssb* gene, plasmids pAL4 and pAL5 were transformed into KL450, an *ssb-1* (Ts) strain of *E.coli*. Unlike the parental plasmid, pLG284, which carries the intact *ssb* gene, pAL4 and pAL5 failed to suppress the temperature sensitivity of the *ssb-1* strain (Table 3.1). Results of this suppression data and the restriction digest analysis indicated that the plasmid *ssb* gene had been disrupted in pAL4 and pAL5.

3.4. Construction of a *lacZ* fusion to *psiB* of pAL1001.

Plasmid pAL1001 carries the *psiB* gene of ColIb on a 2.7 kb *Sau3A* fragment in the pHG165 vector (Fig. 3.3). Nucleotide sequence analysis had identified a *Pst*I site located 29 bp into the coding region of *psiB* (section 2.7). The strategy adopted for creating a *psiB-lacZ* transcriptional fusion was to introduce the 4.7 kb *lacZ*-Km^r cassette into the *Pst*I site internal to the *psiB* gene. However, there is also a *Pst*I site located in the pHG165 vector portion of pAL1001 and so a partial *Pst*I digest had to be performed to linearize pAL1001 as a prerequisite for introduction of the *lacZ*-Km^r cassette.

Conditions of *Pst*I digestion were chosen such that molecules linearized at either *Pst*I site were the major reaction product. Molecules in which digestion had gone to completion (i.e cleavage at both *Pst*I sites had occurred) could be identified and eliminated at a later stage. For the partial digest of pAL1001, 3 µg of DNA were digested with 0.4 units of restriction enzyme for 10 minutes. The reaction volume was 100 µl and included 4 µl ethidium bromide (500 µg ml^{-1}). The inclusion of ethidium bromide (EtBr) into the reaction apparently favours the production of a single cut (Barany, 1985; Osterlund *et al.*, 1982). A conformational change in the DNA which interferes with the enzyme recognition site is brought about by intercalation

Table 3.1. Suppression of the temperature sensitivity of KL450 by plasmids.

Plasmid present	KL450 survival*
None	5.7×10^{-5}
ColIb	3.9×10^{-5}
ColIb*drd-1*	0.79
pLG284	0.95
pAL4	3.4×10^{-5}
pAL5	3.9×10^{-5}
pAL6	4.2×10^{-5}
pAL7	3.8×10^{-5}
pAL12	4.3×10^{-5}

* Colony formation at 44°C relative to that at 30°C.

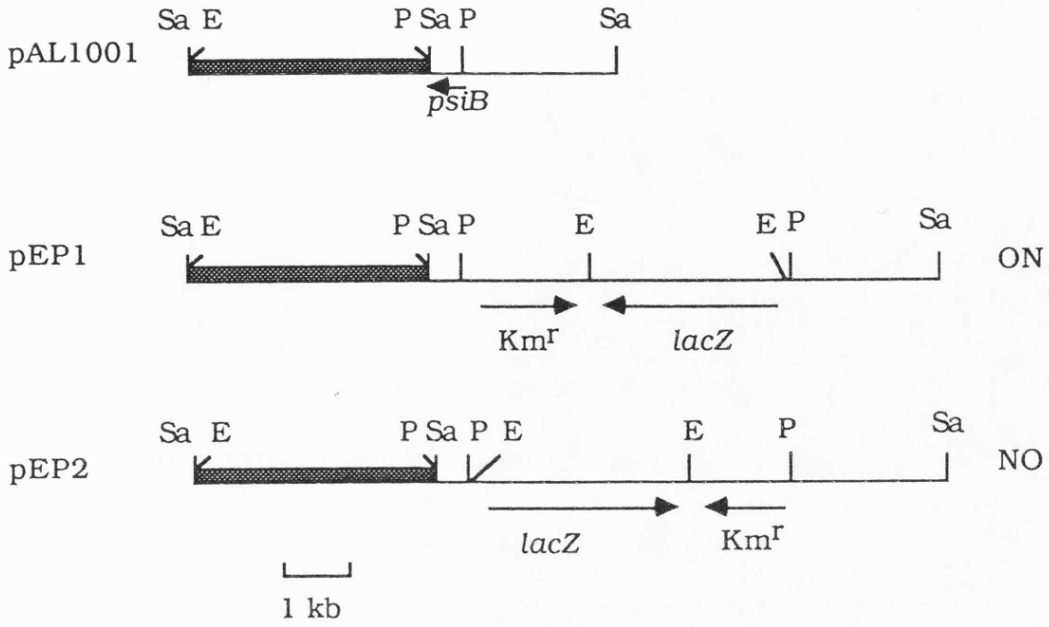


Fig. 3.3. Introduction of the *lacZ*-Km^r cassette into the *psiB* gene of pAL1001.

Line 1 (top) shows the location and direction of transcription of the *psiB* gene on pAL1001. Lines 2 and 3 show pEP1 and pEP2, which have the *lacZ*-Km^r cassette introduced at a *Pst*I site within the *psiB* gene in the ON and NO orientation, respectively. Restriction sites are *Eco*RI (E), *Pst*I (P), *Sal*I (S) and *Sau*3A (Sa).

Restriction fragments were as follows:

	Enzyme	Fragment sizes (kb)	Total (kb)
a) pAL1001	*Pst*I	5.6, 0.43	6.0
	*Eco*RI	6.0	6.0
	*Sal*I	6.0	
b) pEP1	*Pst*I	5.6, 4.7, 0.43	10.7
	*Eco*RI	5.4, 3.0, 2.3	
	*Sal*I	10.7	
c) pEP2	*Pst*I	5.6, 4.7, 0.43	10.7
	*Eco*RI	4.0, 3.7, 3.0	
	*Sal*I	10.7	

between the bases. Such intercalation occurs more readily in linear DNA molecules than in covalently closed plasmid molecules. The digestion reaction was stopped by phenol extraction and ethanol precipitation prior to ligation.

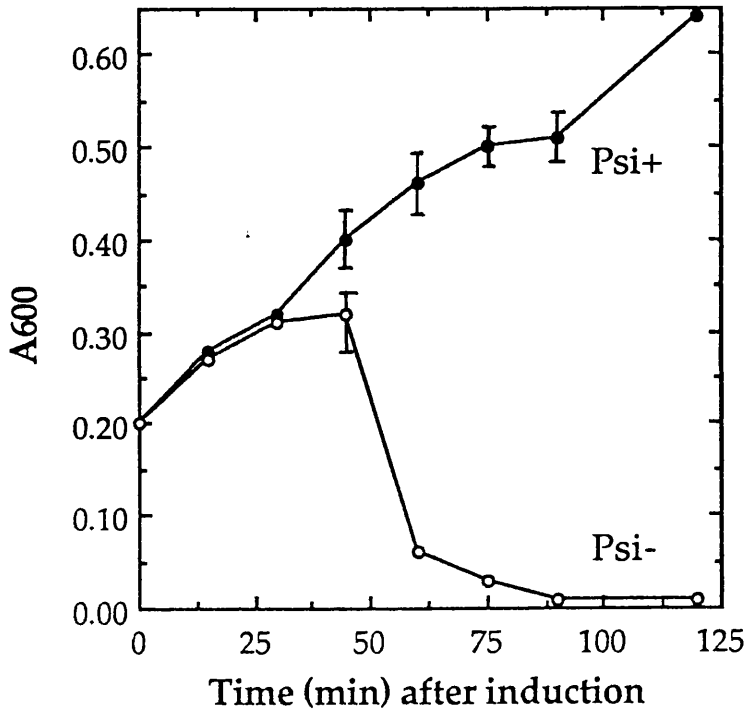
Ligation was carried out using partially *Pst*I digested pAL1001 and the 4.7 kb *lacZ*-Km^r cassette released from pKOK6 by *Pst*I digestion. Recombinant transformants were selected for using the Km^r determinant of the insert.

As there are two *Pst*I sites in pAL1001 and two possible orientations for the insert, four potential recombinant plasmids were attainable (assuming that pAL1001 had only been linearized and not completely digested). All of the combinations would give different products following restriction enzyme digestion with *Eco*RI, such that the orientation and location of the insert could be determined. Figure 3.3 shows restriction maps of two plasmids, pEP1 and pEP2, representative of recombinants with the *lacZ*-Km^r insert located within the *Pst*I site in *psiB* in the ON and NO orientation respectively.

To confirm that the inserts had disrupted the plasmid *psiB* gene, pEP1 and pEP2 were tested for Psi function in JM12 λ$^+$. The parental plasmid, pAL1001, prevented induction of the λ prophage at the restrictive temperature whereas pEP1 and pEP2 did not (Fig. 3.4.). This confirms that the *lacZ*-Km^r insert had disrupted the *psiB* gene and that the *Pst*I site lies within the gene specifying the Psi$^+$ phenotype. It was not possible to test pEP1 and pEP2 for the Psi phenotype in the GY7221 and GC4597 strains, as these tests rely on measurements of β-galactosidase specified by a *sfiA::lacZ* fusion (see section 2.1). β-galactosidase specified by the *psiB-lacZ* transcriptional fusions in the ON orientation would confuse interpretation of the Psi test. The λ induction test was deemed reliable and suitable for this confirmational experiment.

Fig 3.4. λ induction in *recA441* cells.

Effect of plasmids on prophage λ induction in a *recA441* host. At time 0, cells growing at 30°C were shifted to 42°C, and adenine added to the medium to 0.5 mM. Strains carrying pAL1000, pAL1001 or pAL12 showed no cell lysis and the plasmids were designated Psi$^+$ (closed circles). Strains containing no plasmid, pEP1 of pEP2 lysed approximately 60 minutes after temperature shift and the plasmids were designated Psi$^-$ (open circles). Error bars show the upper and lower A_{600} values obtained.



3.5. Construction of a *sog-lacZ* transcriptional fusion in pAL13.

To allow comparison of the regulation of the *ssb* and *psiB* genes with that of a representative transfer gene, a *lacZ* transcriptional fusion was created to *sog*. The *sog* gene has an approximate coding region of 3.5 kb and the smallest recombinant plasmid containing the *sog* gene available at the commencement of this work was pLG215 (Fig. 3.5). This plasmid is a pBR325 derivative carrying an 8.3 kb *Eco*RI fragment from ColIb (Wilkins *et al.*, 1981).

A 4.7 kb *Bgl*II fragment from pLG215 carrying most of the *sog* gene was inserted into the *Bam*HI site of a pUC19 derivative (Fig. 3.5). The pUC19 vector for this construct lacked the *Eco*RI site in the polylinker, which was removed by a T4 DNA polymerase fill in reaction and blunt end ligation. Removal of the *Eco*RI site destroys the α-complementation test for detecting recombinants and therefore colonies containing the desired construct were screened by colony hybridization using the 4.7 kb *Bgl*II fragment from pLG215 as a probe. Plasmid pAL10 was representative of the recombinant plasmids identified by colony hybridization as containing the 4.7 kb *Bgl*II fragment (Fig. 3.5).

The ColIb DNA contained in pAL10 has four *Pst*I sites, three of which were known to lie within the *sog* coding region and could be used for introduction of the *lacZ*-Km^r insert (Bettina Strack, personal communication; Fig. 3.5.). However, it was decided to subclone a region from the 4.7 kb *Bgl*II fragment, to reduce the number of *Pst*I sites and facilitate introduction of the insert.

*Eco*RI sites were introduced into pAL10 by the method of Tn*1732* mutagenesis (Ubben and Schmitt, 1986). This transposon specifies kanamycin resistance and has two *Eco*RI sites located 15 bp from each end (Schoffl *et al.*, 1981). RU4406, which carries Tn*1732*, was transformed with

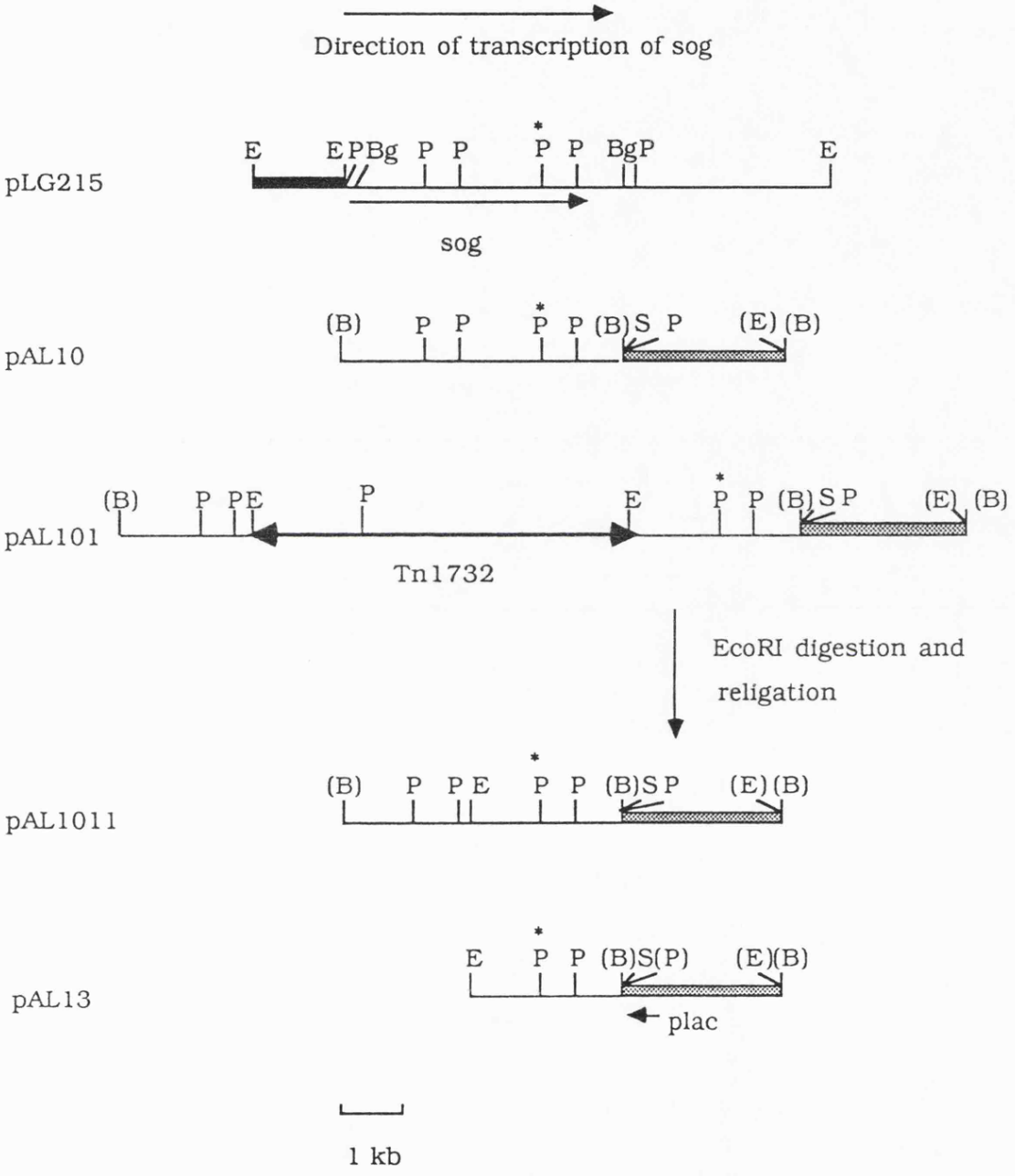
Fig 3.5. Construction of *sog* subclones and introduction of an *Eco*RI site into the *sog* coding region.

Line 1 (top) shows a restriction map of pLG215 and the direction of transcription of the *sog* gene. Lines 2 to 5 show the steps involved in the creation of pAL13. pAL10 (line 2) carries a 4.7 kb *Bgl*II fragment from pLG215 in a pUC19-derived vector. pAL101 (line 3) is a Tn*1732* insertion mutant of pAL10. The Tn*1732* insertion was removed by *Eco*RI digestion and religation to produce pAL1011 (line 4). A 2.3 kb *Eco*RI/*Sal*I fragment from pAL1011 was inserted into *Eco*RI/*Sal*I cleaved pUC19 (with the *Pst*I site in the polylinker removed) to produce pAL13. The position and orientation of the *lacZ* promoter (plac) of the pUC19 vector is shown but the vector portions are not to scale. Direction of transcription of the *sog* gene is from left to right throughout. The asterixed *Pst*I site is used for the introduction of the *lacZ*-Km^r cassette. Restriction sites are *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Pst*I (P) and *Sal*I (S).

Restriction fragment sizes were as follows:

	Enzyme	Fragment sizes (kb)	Total (kb)
a) pAL10	*Pst*I	4.1, 1.4, 0.54, 0.5, 0.48	7.0
	*Sal*I	7.0	
b) pAL101	*Eco*RI	7.0, 6.7	13.7
	*Pst*I	5.9, 4.1, 2.2, 0.54, 0.5, 0.48	
	*Eco*RI/*Sal*I	6.7, 4.7, 2.3	
c) pAL1011	*Eco*RI	7.0	7.0
	*Pst*I	4.1, 1.4, 0.54, 0.5, 0.48	
	*Eco*RI/*Sal*I	4.7, 2.3	
d) pAL13	*Eco*RI/*Sal*I	2.7, 2.3	5.0
	*Pst*I	4.5, 0.5	

Fig.3.5.


Direction of transcription of sog
pLG215
E
E P Bg P P
*
P P Bg P
E
sog
pAL10
(B)
P P
*
P P (B) S P
(E) (B)
pAL101
(B)
P P E
P
E
*
P P (B) S P
(E) (B)
Tn1732
EcoRI digestion and
religation
pAL1011
(B)
P P E
*
P P (B) S P
(E) (B)
pAL13
E
*
P P (B) S (P)
(E) (B)
plac
1 kb

pAL10 with selection for Ap^r specified by the plasmid. Colonies were left at 28°C for 72 hours. This is the optimum temperature for Tn*1732* transposition. Approximately 150 colonies were then pooled, plasmid DNA prepared and used to transform JM83 to Ap^rKm^r. All strain manipulations were now carried out at 37°C, as at this temperature transposition is reduced. Plasmids carrying both Ap^r and Km^r were then analysed for the location of the transposon insertion. Derivatives of pAL10 were obtained with Tn*1732* insertions located throughout the plasmid, but only pAL101 (Fig. 3.5) was used further. The Tn*1732* transposon was removed from this construct by digestion with *Eco*RI and religation, explaining the need for prior destruction of the *Eco*RI site from the vector. Colonies were screened for kanamycin sensitivity to indicate loss of the transposon and pAL1011 is representative of one such Ap^rKm^s plasmid (Fig. 3.5). Restriction digests confirmed that an *Eco*RI site had been introduced into pAL1011 at the site indicated in figure 3.5.

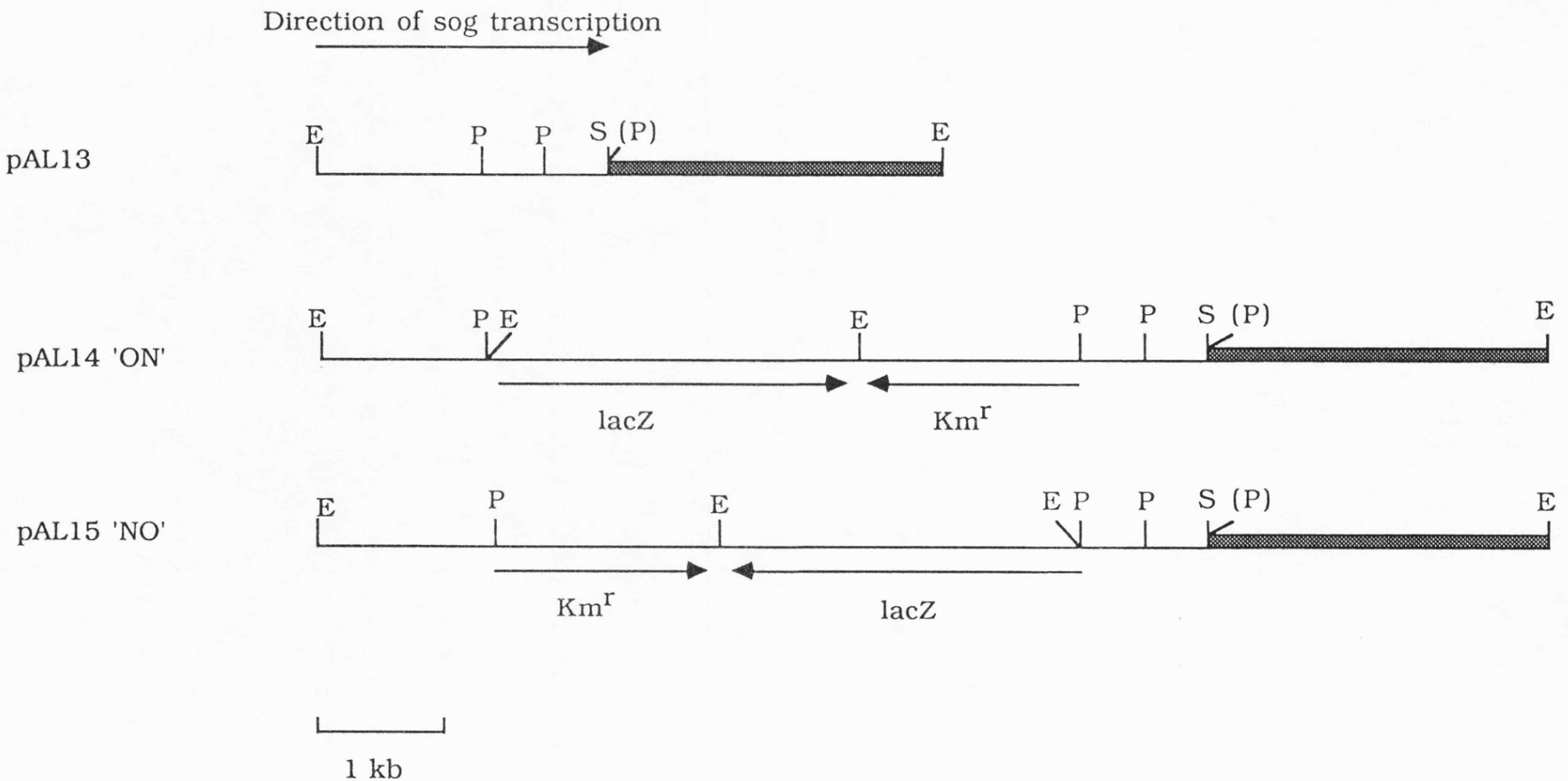
The construction of pAL1011 allowed a 2.3 kb *Eco*RI-*Sal*I fragment containing the 3′ terminal half of the *sog* gene to be inserted into *Eco*RI-*Sal*I cleaved pUC19 vector. This pUC19 vector lacked the *Pst*I site from the polylinker, which was removed by T4 DNA polymerase exonuclease reaction followed by blunt end ligation, thereby reducing the final number of *Pst*I sites. The removal of the *Pst*I site also destroys the α-complementation method of selecting for insertions. However, as this ligation reaction involved fragments with two different ends, a high proportion of ligation products were the desired construct and were screened simply by restriction enzyme analysis. Plasmid pAL13 carries the 2.3 kb *Eco*RI/*Sal*I fragment derived from pAL1011 and contains 2 *Pst*I sites (Fig. 3.5). The next aim was to insert the *lacZ*-Km^r cassette (with *Pst*I ends) at the 5′ terminal *Pst*I site (asterixed in Fig. 3.5 and Fig 3.6) to ensure that the insertion is located within the *sog* coding region.

Fig. 3.6. Introduction of the *lacZ*-Km^r cassette into the *sog* DNA of pAL13. Line 1 (top) shows the direction of transcription of the *sog* gene with respect to pAL13. Lines 2 and 3 show pAL14 and pAL15, which have the *lacZ*-Km^r cassette introduced at a *Pst*I site within the *sog* gene in the ON and NO orientation, respectively. Restriction sites are *Eco*RI (E), *Pst*I (P) and *Sal*I (S).

Restriction fragment sizes were as follows:

	Enzyme	Fragment sizes (kb)	Total (kb)
a) pAL13	*Eco*RI	5.0	5.0
	*Sal*I	5.0	
	*Pst*I	4.5, 0.5	
b) pAL14	*Sal*I	9.7	9.7
	*Pst*I	4.7, 4.5, 0.5	
	*Eco*RI	5.3, 3.0, 1.4	
c) pAL15	*Sal*I	9.7	9.7
	*Pst*I	4.7, 4.5, 0.5	
	*Eco*RI	3.7, 3.0, 3.0	

Fig.3.6.


Direction of sog transcription
pAL13
E
P
P
S (P)
E
pAL14 'ON'
E
P E
E
P
P
S (P)
E
lacZ
Km^r
pAL15 'NO'
E
P
E
E P
P
S (P)
E
Km^r
lacZ
1 kb

The method for introducing the 4.7 kb *lacZ*-Km^r cassette into this fragment of the *sog* gene was identical to that for the *psiB* gene, since pAL13 has two *Pst*I sites. A partial *Pst*I digest of pAL13 was performed to obtain linear molecules and used in a ligation reaction with the 4.7 kb *Pst*I *lacZ*-Km^r fragment. Transformants containing recombinant molecules with the desired insertion were identified using the Km^r determinant. As in the case of insertions in the *psiB* gene, there are four possible recombinant plasmids obtainable if pAL13 is cleaved only once. Fig. 3.6 shows *EcoR*I digests of 12 Km^r recombinants demonstrating 3 of the 4 possibilities. Plasmids giving unexpected digestion products were not characterised further.

Plasmids pAL14 and pAL15 have the ON and NO orientation respectively with the *lacZ*-Km^r insert located within *sog* (Fig.3.8). As pAL14 and pAL15 were derived from pAL13, which only encodes the 3′ region of *sog* there was no functional test available to confirm that the *sog* gene had been disrupted.

3.6. Determination of β-galactosidase levels specified by the *lacZ* fusions to the cloned *ssb* and *psiB* genes.

MC4100 was transformed with pAL4 (*ssb*::ON), pAL5 (*ssb*::NO), pEP1 (*psiB*::ON) and pEP2 (*psiB*::NO). This strain has a deletion of the chromosomal *lac* operon and so can be used as the host strain for β-galactosidase measurements specified by the fusions (Table 3.2).

Only the constructs in the 'ON' orientation gave detectable levels of β-galactosidase. The values do not provide much useful information at this stage, as the constructs contain fragments of ColIb in multicopy vectors. However, an indication of the level of activity required for the production Psi$^+$ phenotype can be gained from the pEP1 value of ~500 units/ mg protein. pEP1 is derived from pAL1001 which gives a detectable Psi$^+$

Table 3.2. β-galactosidase specified by cloned ColIb *ssb*- and *psiB-lacZ* fusions.

Plasmid	Description	β-galactosidase in MC4100*
None		0
pAL4	*ssb*::ON	104
pAL5	*ssb*::NO	0
pEP1	*psiB*::ON	498
pEP2	*psiB*::NO	0

*Values are units mg^{-1} protein and are the means of at least three experiments.

phenotype. This value is a useful reference when considering the activities of the *psiB::lacZ* fusions in ColIb and ColIb*drd-1* plasmids described in Chapter 5. β-galactosidase specified by the *sog*::ON and *sog*::NO fusions was not tested.

3.7. Introduction of the cloned ColIb *ssb-*, *psiB-*, and *sog-lacZ*-Km^r inserts into ColIb and ColIb*drd-1*

As a prerequisite to analysing the expression of the ColIb *ssb*, *psiB* and *sog* genes, it was necessary to introduce the *lacZ*-Km^r fusions constructed in the cloned genes into ColIb and ColIb*drd-1*. The method used is essentially as described by Rees *et al.* (1987) and involves gene replacement via homologous recombination. A recombinant plasmid, carrying a selectable mutation within a cloned fragment, derived from the target replicon is first linearized by restriction endonuclease digestion. This DNA is then introduced by transformation into JC7623, an *E. coli recBC sbcB* derivative carrying the target replicon. This strain lacks exonuclease V activity, preventing degradation of the linearized plasmid, while the *sbcB* mutation, which inactivates exonuclease I, restores the recombinational activity eliminated by the *recBC* mutation. Linear molecules can only be maintained via integration into another replicon by a double recombination event. Thus a selectable mutation is essential. The inserts generated within the cloned *ssb*, *psiB* and *sog* genes contained the Km^r gene to allow for selection of the gene replacement event.

3.8. Construction of ColIb *ssb-lacZ* insertion mutants

Following the scheme outlined above, JC7623 (ColIb) and JC7623 (ColIb*drd-1*) were transformed with pAL4 and pAL5, containing the cloned *ssb* ON and NO fusions, respectively. These plasmids were previously linearized at a unique *Sal*I site located in the vector portion of the

constructs. Thus, four separate transformations were performed. The kanamycin resistant transformants obtained were screened for chloramphenicol sensitivity, which would indicate the absence of the recombinant plasmid, pAL4 or pAL5. Plasmid DNA was prepared from chloramphenicol sensitive transformants and analysed by restriction enzyme digest to confirm that insertion had occurred at the correct target site.

The *lac*Z-Km^r insert contains no *Sal*I sites but has two *Eco*RI sites (Fig. 3.1). An increase in size from 10.1 kb to 14.8 kb for the S4 *Sal*I fragment was observed for all the Km^{r}Cm^s plasmids. The *Eco*RI digest pattern changed such that the E1 band which runs as a doublet band with E2 in *Eco*RI digests of ColIb and ColIb*drd-1* is cleaved to give bands of 16 kb, 6 kb and 3 kb for the ON fusions and 14.4 kb, 7.6 kb and 3 kb for the NO fusions. These digestion products are as expected for the introduction of the *lac*Z-Km^r cassette into the ColIb *ssb* gene (Fig. 3.7). The 3 kb *Eco*RI fragment is internal to the *lac*Z-Km^r cassette. The plasmids were designated pAL6 (ColIb*drd-1 ssb*::ON), pAL7 (ColIb*drd-1 ssb*::NO), pAL8 (ColIb *ssb*::ON), and pAL9 (ColIb *ssb*::NO).

A Southern hybridization experiment was performed using the cloned ColIb *ssb* gene present on a 3.95 kb *Cla*I-*Sal*I fragment from pLG284 as a probe, to confirm that the ColIb *ssb* gene had been disrupted in pAL6, pAL7, pAL8, and pAL9. Figure 3.8 shows the alteration of the hybridization pattern of the *ssb* probe to *Eco*RI digests of pAL6, 7, 8, and 9 compared to an *Eco*RI digest of ColIb. The *ssb* probe hybridizes to *Eco*RI fragments of 16 kb and 6 kb from pAL6 and pAL8 and 14.4 kb and 7.6 kb from pAL7 and pAL9. The difference in *Eco*RI hybridization pattern of pAL6 and 8 compared to pAL7 and 9 is due to the different orientations of the *lac*Z-Km^r cassette. A genetic test involving suppression of the temperature sensitivity of an *ssb-1*

Fig. 3.7. Expected *Eco*RI hybridization products for ColIb *ssb*::ON and NO insertions using a 3.95 kb *Cla*I/*Sal*I fragment of pLG284 as a probe. The E1 (*Eco*RI) fragment of ColIb is shown with the *lacZ*-Km^r cassette introduced into a *Pst*I site within the 5′ region of *ssb*. The *Eco*RI sites of the *lacZ*-Km^r cassette are shown above the line for the ON orientation (pAL6 and pAL8) and below for the NO orientation (pAL7 and pAL9). The extent of the DNA covered by the probe is shown as a thick black line. Double headed arrows are used to illustrate the expected *Eco*RI hybridization products. Restriction sites are *Eco*RI (E), *Pst*I (P), *Sal*I (S).

Fig.3.7.

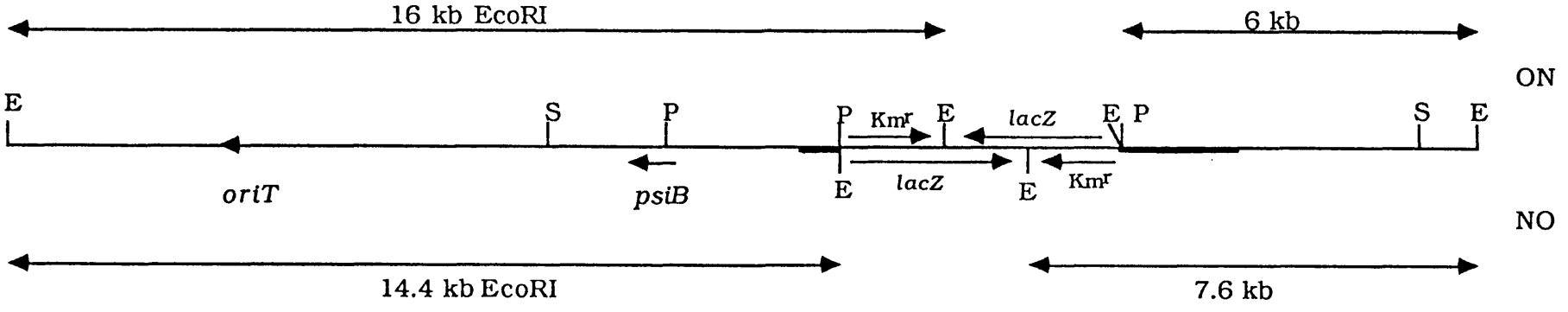

16 kb EcoRI
6 kb
ON
E
S
P
P
Km^r
E
lacZ
E
P
S
E
oriT
psiB
E
lacZ
E
Km^r
NO
14.4 kb EcoRI
7.6 kb

Fig. 3.8. Results of a Southern hybridization using a 3.95 kb *Cla*I/*Sal*I fragment from pLG284 carrying the intact *ssb* gene as a probe.

Lanes correspond to (1) pAL9 x *Eco*RI, (2) pAL8 x *Eco*RI, (3) pAL7 x *Eco*RI, (4) pAL6 x *Eco*RI, (5) ColIb x *Eco*RI separated by agarose gel electrophoresis.

strain of *E. coli* is described at the end of this chapter, which aids confirmation that the *ssb* gene in pAL6 and pAL7 is disrupted.

3.9. Construction of ColIb *psiB-lacZ* insertion mutant

Plasmids pEP1 and pEP2 (*psiB* 'ON' and 'NO' respectively) were linearized at a unique *Sal*I site in the pHG165 polylinker and used to transform JC7623 (ColIb) and JC7623 (ColIb*drd-1*) to kanamycin resistance. The colonies obtained were screened for ampicillin sensitivity to indicate the absence of the recombinant plasmid (pEP1 or pEP2). Colonies which were Km^rAp^s were picked, plasmid DNA prepared and analysed by restriction enzyme digestion (data not shown). Plasmids pEP3 and pEP4 are representative of the ColIb*drd-1* derivatives with the *lacZ*-Km^r cassette inserted in the 'ON' and 'NO' orientations respectively. Plasmid pEP5 is representative of the derivatives of ColIb with the insert in the ON orientation, a corresponding ColIb *psiB*::NO derivative was not discovered. An increase in size from 10.1 kb to 14.8 kb of the S4 fragment was observed for pEP3, pEP4, and pEP5. The E1 fragment was cleaved by *Eco*RI digestion to give fragments of 13 kb, 9.1 kb and 3 kb for pEP3 and pEP5 (ON orientation) and 11.2 kb, 10.6 kb and 3 kb for pEP4 (NO orientation). These digest patterns are as expected if the ColIb *psiB* gene has been disrupted by the appropriately orientated insert (Fig. 3.9).

A Southern hybridization experiment was performed using the intact *psiB* gene carried on a 2.7 kb *Sau*3A fragment from pAL1001 to confirm that the ColIb *psiB* gene had been disrupted. The probe hybridized to a 14.8 kb *Sal*I fragment and to *Eco*RI fragments of 13 kb and 9.1 kb for pEP3 and pEP5 and 11.2 kb and 10.6 kb for pEP4 (Fig. 3.10). This data confirms that the *psiB* gene is disrupted.

Fig. 3.9. Expected *Eco*RI and *Sal*I hybridization products for ColIb *psiB*::ON and NO insertions using a 2.7 kb *Sau*3A fragment of pAL1001 as a probe.

The E1 (*Eco*RI) fragment of ColIb is shown with the *lac*Z-Km^r cassette introduced into a *Pst*I site within the 5′ region of *psiB*. The *Eco*RI sites in the *lac*Z-Km^r cassette are shown above the line for the ON (pEP3 and pEP5) orientation and below the line for the NO orientation (pEP4). The extent of the DNA covered by the probe is shown as a thick black line. Double headed arrows are used to illustrate the expected *Eco*RI and *Sal*I hybridization products. Restriction sites are *Eco*RI (E), *Pst*I (P) and *Sal*I (S).

Fig.3.9.

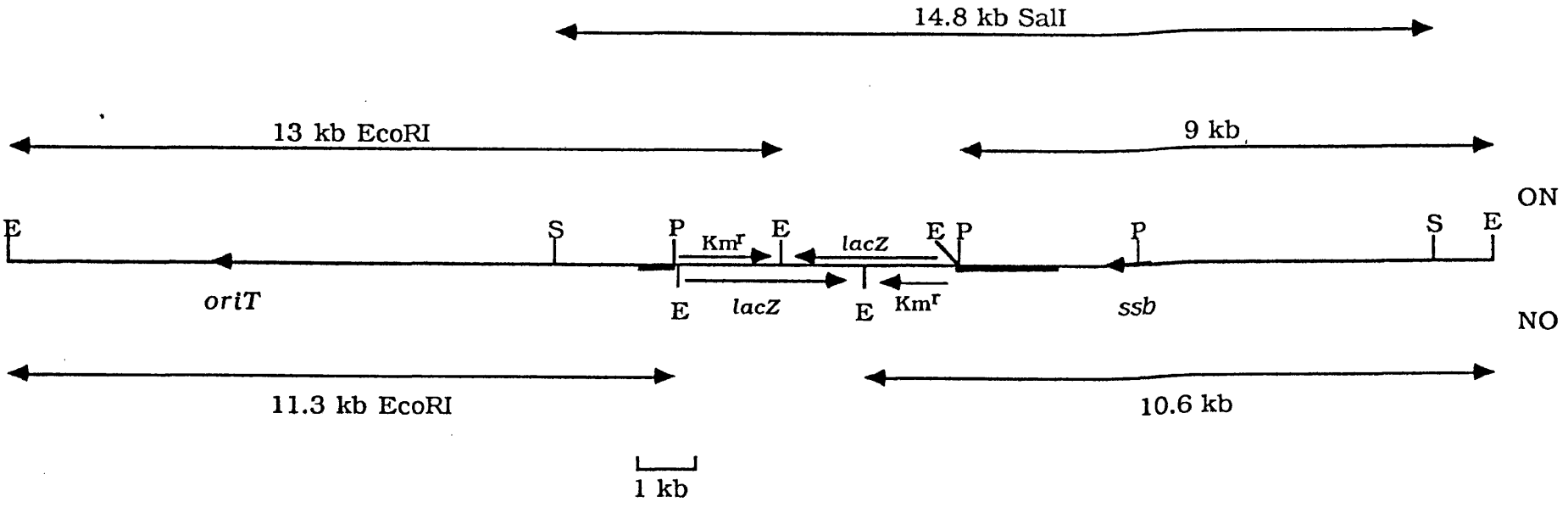
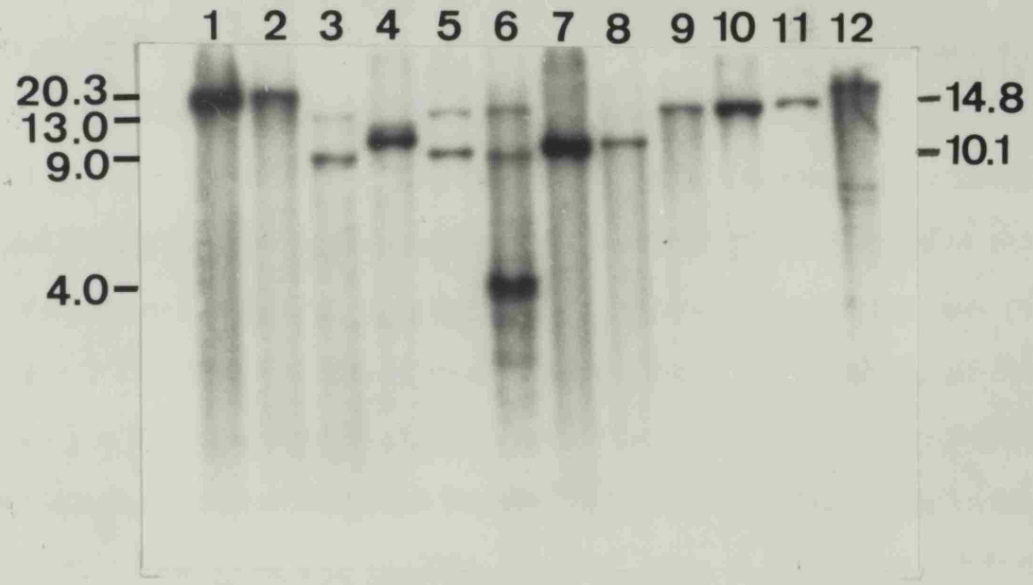

14.8 kb SalI
13 kb EcoRI
9 kb
ON
E
S
P
Km^r
E
lacZ
E
P
P
S
E
oriT
E
lacZ
E
Km^r
ssb
NO
11.3 kb EcoRI
10.6 kb
1 kb

Fig. 3.10. Results of a Southern hybridization using a 2.7 kb Sau3A fragment from pAL1001 carrying the intact *psiB* gene as a probe.

Lanes correspond to (1) ColIb x *Eco*RI, (2) ColIb*drd-1* x *Eco*RI, (3) pEP3 x *Eco*RI, (4) pEP4 x *Eco*RI, (5) pEP5 x *Eco*RI, (6) pAL11 x *Eco*RI, (7) ColIb x *Sal*I, (8) ColIb*drd-1* x *Sal*I, (9) pEP3 x *Sal*I, (10) pEP4 x *Sal*I, (11) pEP5 x *Sal*I and (12) pAL11 x *Sal*I separated by agarose gel electrophoresis.



3.10. Construction of a *psiB-lacZ* transcriptional fusion to *psiB* of pAL12.

Plasmid pLG288 contains the Tn*903*-based Km^r fragment in the *ssb* gene of ColIb*drd-1* and has the ability to confer a Psi$^+$ phenotype on the host cell. The *psiB*::ON fusion expresses Km^r constitutively and so could not be introduced into pLG288 as there was no positive selection available. So that a measurement of the transcriptional activity of *psiB* could be made under similar genetic conditions, a derivative of ColIb*drd-1* was created that expressed the Psi$^+$ phenotype but was Km^s.

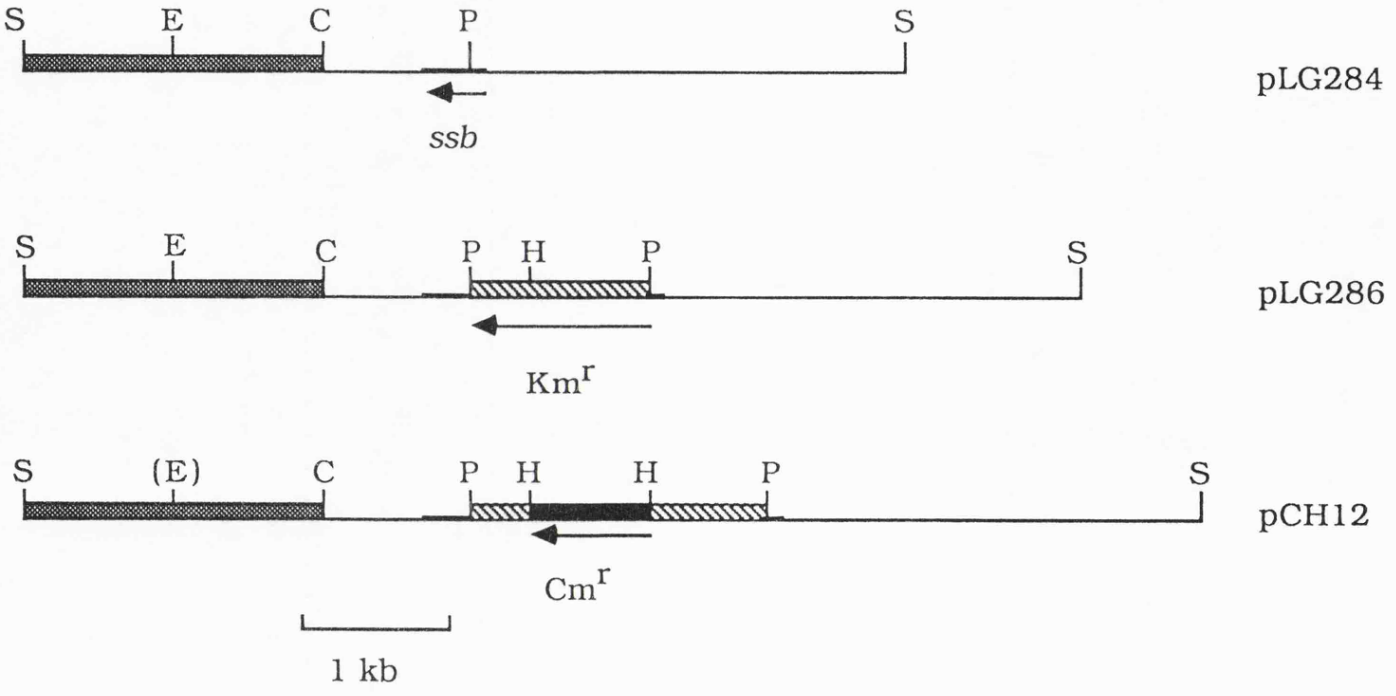
First, a promoterless chloramphenicol acetyltransferase (*cat*) gene present on a 0.792 kb *Hin*dIII-flanked cartridge, was inserted into the *Hin*dIII site within the Km^r gene present in *ssb* of the recombinant plasmid pLG286 (Fig. 3.11). This 'double' insertion (*ssb*::Km::Cm^r) was created by Chris Howland in this laboratory for another line of work. pLG286 contains the 1.2 kb *Pst*I *aphA-1* fragment specifying Km^r inserted into the *Pst*I site of pCH4 (Fig. 3.11). This *Pst*I site is located within the 5′ coding region of the *ssb* gene. The Km^r fragment in pLG286 has a unique *Hin*dIII site into which the promoterless *cat* gene had been inserted to produce pCH12. Prior to the insertion of the *cat* gene into pLG286, the Cm^r gene in the vector portion had been inactivated by digestion with *Eco*RI followed by a T4 DNA polymerase fill in reaction and blunt-ended ligation to produce a frame shift mutation. pCH12 carries the *cat* gene in the same transcriptional orientation as the kanamycin resistance gene and so expresses chloramphenicol resistance (Cm^r) constitutively while inactivating the kanamycin resistance determinant.

The double insertion in *ssb* of pCH12 (*ssb*::Km::Cm^r) was introduced into ColIb*drd-1* by the gene replacement method described above. pCH12 was linearized at a *Sal*I site in the vector portion and used to transform JC7623 (ColIb*drd-1*) to Cm^r. The Cm^r transformants were pooled and used as donors in a conjugation experiment with GI65N recipients with selection

Fig. 3.11. Construction of pCH12.

Line 1 (top) shows a restriction map of pLG284 and the location and direction of transcription of the *ssb* gene. Lines 2 and 3 show pLG286 and pCH12. The Km^r gene is represented by a hatched box and the Cm^r gene as a filled box. The direction of transcription of the antibiotic resistance genes is also shown. Restriction sites are *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P) and *Sal*I (S).

Fig.3.11.



for Nal^{r}Cm^r transconjugants. This would ensure that the Cm^r colonies contained ColIb*drd-1* derivatives as pCH12 is non-mobilisable and therefore should not be transferred.

Plasmid DNA was prepared from the Cm^r transconjugants. Restriction digest analysis and Southern hybridization with the intact ColIb *ssb* gene as a probe were performed to check that the ColIb *ssb* gene had been disrupted. The *ssb* probe hybridised to bands of 15.2 kb and 7.0 kb. pAL12 is representative of the ColIb*drd-1* derivatives which had the Km::Cm^r insert in *ssb*. Section 3.12. of this chapter contains further genetic evidence that the *ssb* gene of pAL12 is disrupted as judged by the inability of the plasmid to suppress the temperature sensitivity of KL450, an *ssb-1* strain of *E. coli*.

pAL12 (ColIb*drd-1 ssb*::Km::Cm^r) was tested in GY7221, GC4597 and JM12 λ^+ for the ability to confer a Psi$^+$ phenotype. These Psi functional tests are identical to those described in Chapter 2, section 2.1 and involved *recA441* and *recA730* mutant strains. Table 3.3 and Fig. 3.4. show that pAL12, like pLG288 (ColIb*drd-1 ssb*::Km^r), displays a Psi$^+$ phenotype. Thus, a ColIb*drd-1* plasmid that expressed the Psi phenotype constitutively but was Km^s had been created.

Next, the cloned *psiB*::ON fusion in pEP1 was introduced into pAL12 using Km^r as positive selection for the gene replacement. pEP1 was linearized at the unique *Sal*I site and used to transform JC7623 (pAL12) to Km^r. The Km^r colonies were tested for Ap^s to confirm that pEP1 had not been maintained. Plasmid DNA was prepared from the Km^{r}Cm^{r}Ap^s colonies and analysed by restriction enzyme analysis and Southern hybridization using the intact *psiB* gene carried on the 2.7 kb *Sau3*A fragment from pAL1001 as a probe to check for gene disruption. The *psiB* probe hybridised to a *Sal*I fragment of 16.7 kb and to *Eco*RI fragments of 13 kb and 4 kb. pAL11 is representative of the derivatives of pAL12 containing the *psiB*::ON fusion. This plasmid enabled measurement of the

Table 3.3. Effect of plasmids on *sfiA::lacZ* expression in *recA441* and *recA730* cells.

Plasmid	β-galactosidase in strain*	
	GC4597	GY7221
None	2320	2997
ColIb*drd-1*	1700	2780
pLG288	120	230
pAL12	145	285

* Values are units mg^{-1} measured in GC4597 (*recA441*) after 1 hour at 42°C in the presence of 0.5 mM adenine and in GY7221 (*recA730*) after growth at 37°C. Each value is the mean of at least three experiments.

transcriptional activity of *psiB* to be made under conditions in which an insert in *ssb* was apparently affecting the expression of *psiB*. The results of such measurements are described in the following chapter.

3.11. Construction of a ColIb *sog-lacZ* transcriptional fusion

Plasmids pAL14 and pAL15 were linearized at the unique *Sal*I site in the vector portion and used to transform JC7623 (ColIb) and JC7623 (ColIb*drd-1*) to kanamycin resistance. Transformants were screened for ampicillin sensitivity to check for the absence of pAL14 and pAL15. Plasmids pAL17 and pAL18 are representative of the ColIb and ColIb*drd-1* derivatives with the *lacZ*-Km^r cassette inserted in the *sog* gene in the ON orientation. Plasmid pAL19 is a derivative of ColIb*drd-1* with the insert in the NO orientation.

Confirmation that the *lacZ*-Km^r insert had disrupted the *sog* gene was achieved by restriction enzyme analysis and Southern hybridization using the 4.7 kb *Bgl*II fragment from pAL10 as a probe. This fragment contains sequence that lie on either side of the *Pst*I site used in construction of the *lacZ*-Km^r inserts.

Figure 3.12 shows the results of the Southern hybridization The 4.7 kb *Bgl*II fragment hybridized to *Eco*RI fragments of 13 kb for ColIb and ColIb*drd-1*, 6.7 kb and 3.3 kb for pAL17 and pAL18 and *Eco*RI fragments of 5.1 kb and 4.9 kb for pAL19. Figure 3.13 shows that these are the expected fragments for the introduction of the *lacZ*-Km^r cassette into the *sog* gene.

3.12. Phenotypic changes associated with the *ssb-* and *sog-lacZ* transcriptional fusions.

The results of restriction mapping and Southern hybridization experiments described above indicate that the *lacZ*-Km^r cassette had been inserted into the *ssb*, *psiB* and *sog* genes of ColIb and ColIb*drd-1*. By default,

Fig. 3.12. Expected *Eco*RI hybridization products for ColIb *sog*::ON and NO insertions using a 4.7 kb *Bgl*II fragment from pLG215 as a probe.

The E3 (*Eco*RI) fragment of ColIb is shown with the *lacZ*-Km^r cassette introduced into a *Pst*I site within the *sog* gene. *Eco*RI sites in the *lacZ*-Km^r cassette are shown above the line for the ON orientation (pAL17 and pAL18) and below for the NO orientation (pAL19). The extent of the DNA carried by the probe is represented by a thick black line. Double headed arrows are used to illustrate the expected *Eco*RI hybridization products. Restriction sites are *Eco*RI (E), *Bgl*II (B) and *Pst*I (P).

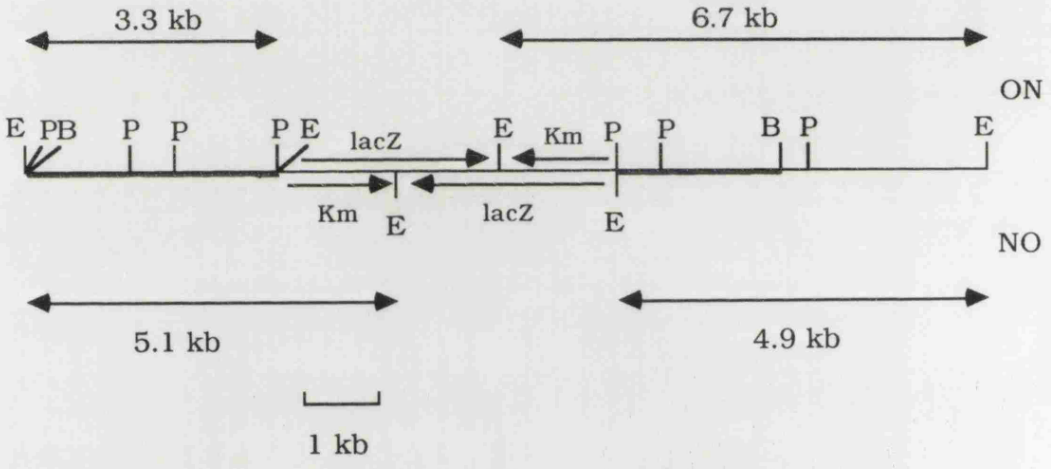
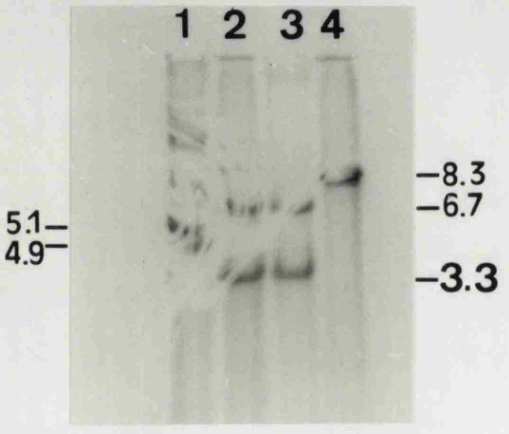


Fig. 3.13. Results of a Southern hybridization using a 4.7 kb *Bgl*II fragment from pLG215 carrying the intact *sog* gene as a probe.

Lanes correspond to (1) pAL19 x *Eco*RI, (2) pAL18 x *Eco*RI, (3) pAL17 x *Eco*RI and (4) ColIb x *Eco*RI separated by agarose gel electrophoresis.



this procedure had also created insertion mutants of these genes. Some properties of the ColIb*drd-1 psiB*::*lacZ*-Km^r mutants pEP3 and pEP4 are described in chapters 4 and 5. The following sections describe phenotypic changes associated with the *ssb* and *sog* insertion mutants, which should aid confirmation that the genes had been disrupted and possibly provide additional information on their role.

3.13. Properties of pAL6 and pAL7

Presence of ColIb*drd-1* in an *E. coli ssb-1* (Ts) strain suppresses the temperature sensitivity to almost 80%. Plasmids pAL6 and pAL7 (ColIb*drd-1 ssb* 'ON' and 'NO' respectively) were analysed for their ability to suppress the temperature sensitivity of KL450 (*ssb-1*). Plasmids pAL8 and pAL9 are derived from ColIb (wild type), which is unable to suppress the *ssb-1* phenotype and so were not analysed in these functional tests.

The data shown in table 3.1 indicate that the *ssb* gene in pAL6 and pAL8 has been disrupted, as these plasmids unlike the parental ColIb*drd-1* no longer show the suppressing ability.

3.13. Properties of pAL18 and pAL19

Presence of ColIb*drd-1* in a *dnaG* (Ts) strain will result in almost total suppression of the temperature sensitivity. Plasmids pAL18 and pAL19 (ColIb*drd-1 sog* 'ON' and 'NO' respectively) were analysed for their ability to suppress the temperature sensitivity of BW86, to demonstrate Sog primase activity, and for their ability to form transconjugants. Plasmid pAL17 is derived from ColIb (wild type), which does not express the primase activity and forms few transconjugants. Hence, pAL17 was not analysed in these functional tests.

Table 3.4a shows the results of quantitative *dnaG* (Ts) suppression tests. Plasmid pLG250 is a known primase defective mutant of ColIb*drd-1*

Table 3.4a. Suppression of the temperature sensitivity of BW86 by plasmids.

Plasmid present	BW86 survival*
None	$< 10^{-7}$
ColIb*drd-1*	0.89
pAL18	0.84
pAL19	0.83
pLG250	$< 10^{-7}$

* Colony formation at 42°C relative to that at 30°C.

Table 3.4b. Transconjugant formation by a *sog* mutants of ColIb*drd-1*.

Plasmid(s) present	Transconjugants*
pLG221	1.8×10^8
pAL18	2.9×10^3
pAL19	3.5×10^3
pAL18, pLG215	2.4×10^3
pAL18, pLG2020	4.1×10^3
pAL19, pLG215	3.9×10^3
pAL19, pLG2020	2.9×10^3

* Values show the number of Km^rNal^r transconjugants per ml of mating mixture obtained after a 40 min conjugation. Donor strains were plasmid containing MC4100. C600N was the recipient.

and was used as a negative control (Chatfield *et al.*, 1986). Plasmids pAL18 and pAL19, even though they were thought to contain an insert in the *sog* gene, were still able to suppress the temperature sensitivity of BW86 to a similar level as the parental plasmid ColIb*drd-1*. The primase moiety of the Sog polypeptide is encoded by the 5′ portion of the *sog* gene (Boulnois *et al.*, 1979). The fact that pAL18 and pAL19 still suppressed the temperature sensitivity of BW86 indicates that the insert does not destroy primase function. The *Pst*I site at which the *lacZ*-Km^r cassette was introduced is located downstream of the primase-encoding region. Presumably the insert still allows a polypeptide with primase activity to be produced.

In contrast, Table 3.4b shows that pAL18 and pAL19 are highly defective in transconjugant formation. In addition to the primase function, the Sog polypeptides are thought to act as DNA transport proteins, facilitating the passage of DNA from donor to recipient cell (Merryweather *et al.*, 1986). The portion of the protein required for this role is thought to be encoded by the 3′ region of the gene. Thus it is possible that the insertion in the *sog* gene in pAL18 and pAL19 destroys the DNA transport function of the Sog protein but still allows synthesis of an active primase. It is also possible that the insertion in *sog* is having a polar effect on downstream genes in the Tra2/3 region. The *lacZ* and Km^r genes of the insert are orientated towards each other and are separated by a bidirectional transcriptional terminator such that there should be no transcription emanating from the cassette. Such a lack of transcriptional read out would have a profound effect on downstream genes, if *sog* is a member of an operon. Indeed, Table 3.4b shows that the defect in transconjugant formation shown by pAL18 and pAL19 could not be complemented by the presence of a *sog*$^+$ recombinant (pLG215 or pLG2020), which suggests that the insert is having a polar effect.

3.15. Summary

This chapter concerned the construction of *lacZ* transcriptional fusions to the *ssb, psiB* and *sog* genes of ColIb and ColIb*drd-1*. A 4.7 kb fragment, containing a promoterless β-galactosidase gene and the kanamycin resistance determinant of Tn*903*, was used to create the fusions: All three genes contain a *Pst*I site internal to the coding sequence into which the *lacZ*-Km^r cassette was inserted. These insertions were initially made in cloned *psiB, ssb* and *sog* genes and constructs carrying the cassette in both possible orientations were created. Each insertion was then transferred back into ColIb and ColIb*drd-1* by a gene replacement strategy using Km^r as a selectable marker. Disruption of the correct target gene was confirmed using restriction digest analysis and Southern hybridization of the ColIb derivatives. For the inserts in *ssb* and *sog* of ColIb*drd-1*, clearly defined genetic tests were also available to aid confirmation of gene disruption. Although the construction of the fusions was a lengthy procedure, the subsequent estimates of transcriptional activity by monitoring β-galactosidase activity was rapid and more flexible than the RNA extraction and northern hybridization techniques originally planned.

Chapter 4.
Regulation of expression of *ssb*, *psiB*, and *sog*.

4.1. Introduction

The work described in the previous chapter involved the creation of *lacZ* transcriptional fusions to the *ssb*, *psiB* and *sog* genes of ColIb and ColIb*drd-1*. These fusions could then be used to investigate regulatory systems controlling expression of these plasmid genes. The aim was to compare expression of two leading region genes (*ssb* and *psiB*) to that of a representative *tra* gene (*sog*). Two broad questions were addressed:

1) *What is the effect on expression of ssb and psiB when the ColIb transfer system is derepressed by a drd mutation?* There is already genetic evidence to suggest that plasmid *ssb* genes are coordinately regulated with the transfer genes (Golub and Low, 1986a; Howland *et al.*, 1989). However, it is not known how highly expressed the *ssb* gene is and whether regulation is at the transcriptional level. Previous work on the expression of *psiB* genes had centred on those of R6-5 and F and had involved the creation of *lacZ* transcriptional fusions in multicopy vectors (Dutreix *et al.*, 1988). By measuring β-galactosidase activity specified by the ColIb and ColIb*drd-1 ssb-* and *psiB-lacZ* fusions, an estimate of transcriptional activity could be obtained.

2) *What is the effect of DNA damaging agents on expression of ssb and psiB?* There is some evidence that the chromosomally-encoded *ssb* gene is a member of the SOS regulon, with expression increased by treatment with DNA damaging agents such as UV-irradiation or mitomycin C in a *recA/lexA* dependent manner (Brandsma *et al.*, 1983). The chromosomal and plasmid-encoded *ssb* genes share ~65% sequence identity and so it was possible that the plasmid *ssb* gene is also under SOS control. The proposed role of PsiB is to prevent triggering of the SOS response during the

conjugative transfer of single-stranded DNA and so *psiB* expression may be increased by SOS inducing treatments.

4.2. The effect of derepression of the ColIb transfer system on expression of *ssb*, *psiB*, and *sog*.

Table 4.1 shows the activity of β-galactosidase specified by the 'ON' fusions in ColIb and ColIb*drd-1*. These 'ON' fusions placed *lacZ* in the same transcriptional orientation as the target gene. As expected, no β-galactosidase was determined when the insertion was in the opposite 'NO' orientation (data not shown). The host strain used was MC4100 which carries a *lac* deletion so that there was no chromosomally encoded enzyme present in the cells. The assay was performed on actively growing cells with selection for kanamycin resistance encoded by the ColIb derivative. These results give an indication of the level of transcriptional activity in an established plasmid-containing strain.

β-galactosidase was undetectable in strains containing ColIb derivatives with the *psiB*- or *ssb-lacZ* 'ON' fusion (pEP5 and pAL8). The corresponding derivatives of ColIb*drd-1*, (pEP3 and pAL6) displayed low, yet consistently detectable activities. The levels of β-galactosidase in strains containing pAL17 and pAL18 (ColIb *sog* 'ON' and ColIb*drd-1 sog* 'ON' respectively) indicate that expression of *sog* is strongly repressed in a wild-type plasmid, but the gene is expressed at a much higher level than *ssb* and *psiB* when the transfer system is derepressed. Thus it appears that *psiB* and *ssb* do show coordinate regulation with the transfer genes, although they are transcribed at a low level. The level of β-galactosidase specified by pEP3 (ColIb*drd-1 psiB* 'ON') was always slightly greater than that determined by pAL6 (ColIb*drd-1 ssb* 'ON').

Table 4.1. β-galactosidase specified by ColIb mutants carrying the *lacZ*-Km^r promoter probe.

Plasmid	Description	β-galactosidase in MC4100*
None		0
pAL8	ColIb *ssb*::ON	0
pAL6	ColIb*drd-1 ssb*::ON	16
pEP5	ColIb *psiB*::ON	0
pEP3	ColIb*drd-1 psiB*::ON	33
pAL11	pAL12 *psiB*::ON	297
pAL11/pLG284	pAL12 *psiB*::ON	
	pACYC184 Ω 3.95 *Cla*I/*Sal*I *ssb*$^+$	301
pAL17	ColIb *sog*::ON	3
pAL18	ColIb*drd-1 sog*::ON	1671

* Values are units mg^{-1} protein and are the means of at least three experiments.

4.3. Level of expression of *psiB* of pAL11.

The construction of pAL11 is described in Chapter 3. pAL11 is derived from pAL12, which is a ColIb*drd-1* plasmid containing an insert in *ssb* that results in the production of a Psi$^+$ phenotype. The insert comprises of a Tn*903* derived Km^r cassette into which a Cm^r gene has been inserted. These antibiotic resistance genes are in the same transcriptional orientation as the *ssb* gene. The *psiB* 'ON' fusion had been introduced into pAL12 producing pAL11, to enable measurement of the transcriptional activity of *psiB* to be made under conditions where *psiB* expression apparently had been affected.

From Table 4.1 it can be seen that pAL11, which has an insert in the *ssb* gene, determined approximately ten-fold more β-galactosidase than pEP3, which has no insert in *ssb*. The presence of a recombinant plasmid, pLG284, which carries the intact *ssb* gene, did not affect this increased level of activity, demonstrating that the insert is *cis*-acting. The value of ~300 μg/ml of β-galactosidase for the *psiB::lacZ* fusion of pAL11 gives another indication of the level of *psiB* expression required for a Psi$^+$ phenotype as the parental plasmid, pAL12, has the ability to confer a Psi$^+$ phenotype. The cloned *psiB::lacZ* fusion of pEP1 gave a value for β-galactosidase accumulation of ~500 μg/ml . The parental plasmid of pEP1, pAL1001 also confers a Psi$^+$ phenotype.

4.4. Determination of conditions for SOS induction.

The effect of SOS inducing conditions on expression of *ssb* and *psiB* was investigated by treating plasmid-containing cells with UV-irradiation, mitomycin C, or nalidixic acid.

For treatment with mitomycin C, the cultures were grown to an OD_{600} of 0.35 and then diluted back to OD_{600} of 0.1. Mitomycin C was added to 1 μg/ml and the culture shaken vigorously in the dark. Samples were

taken for β-galactosidase assay after one hour. A control culture was treated in an identical manner but without addition of mitomycin C.

For UV-irradiated cultures, the cells were grown to an OD_{600} of 0.35 and then diluted to OD_{600} of 0.05. The cultures were then treated with UV-irradiation (10 J m^{-1}) and samples taken for β-galactosidase assay after a one hour incubation at 37°C. A control culture was treated in an identical manner but without the treatment with UV-irradiation.

For treatment with nalidixic acid, the cells were grown as described above and nalidixic acid added to 40 μg/ml after dilution of the culture. The culture was incubated for a further one hour and samples taken for β-galactosidase assay. All cultures were grown in SGC medium at 37°C unless otherwise stated.

In order to confirm that the treatments described above were sufficient to produce an SOS inducing signal, an initial control experiment was performed using GC4415. This strain is *recA*$^+$ and carries a *sfiA::lacZ* fusion. The *sfiA* gene is under SOS control and so triggering of the SOS response can be monitored by measuring β-galactosidase activities following inducing treatments (Huisman and D'Ari, 1981). Table 4.2 shows the level of β-galactosidase specified by GC4415 before and after treatment with mitomycin C, UV-irradiation or nalidixic acid. All three treatments resulted in the increased accumulation of β-galactosidase, although treatment with mitomycin C or UV-irradiation appeared to be a stronger inducing treatment. These treatments produced 36 and 43-fold increases in *sfiA* expression, respectively, whereas nalidixic acid treatment only resulted in an eight-fold increase. It was concluded from these results using *sfiA* that the conditions employed would be suitable to determine whether *ssb* and *psiB* are subject to the same type of control.

Table 4.2. Effect of SOS inducing treatments on *sfiA::lacZ* expression in GC4415 (*recA*+) cells.

β-galactosidase in GC4415*			
No treatment	+ UV	+ Mitomycin C	+ Nalidixic acid
25	1163	988	210

* Values are units mg^{-1} protein and are the means of at least three experiments.

Treatments were: UV irradiation, 10 J m^{-2}; mitomycin C, 1 μg/ml; and nalidixic acid, 40 μg/ml. β-galactosidase was determined after a 1 h incubation.

4.5. Effect of SOS inducing treatments on expression of *ssb* and *psiB* in *recA*$^+$ cells.

The *ssb* 'ON' fusions, pAL6 and pAL8, and the *psiB* 'ON' fusions, pEP3 and pEP5 were used to investigate whether expression of these genes can be increased by the SOS inducing treatments described above. Also included in these studies was the ColIb*drd-1 sog* 'ON' fusion, pAL18 and pAL11. The host strain used was MC4100, which is *recA*$^+$/*lexA*$^+$, and therefore genes under *recA/lexA* control should be inducible. Table 4.3a shows the level of β-galctosidase specified by the 'ON' fusions, with and without SOS inducing treatments. The data indicates that *ssb* and *psiB* expression is increased by treatment with UV-irradiation and mitomycin C but not by nalidixic acid. Expression of the *sog* gene is not affected by such treatments indicating that the induction is not a general feature of all plasmid-encoded genes. Expression of *psiB* on pAL11 was also not affected by the treatments.

The level of induction of *ssb* and *psiB* is greatest when the plasmids carried a *drd* mutation. Induction of *ssb* and *psiB* on wild-type ColIb was slightly greater following UV-irradiation than mitomycin C treatment. Thus it appears that *ssb* and *psiB* may be subject to SOS control even though there was no induction by nalidixic acid. It was thought possible at this preliminary stage, that if these genes are under LexA repression, they are strongly repressed and the nalidixic acid treatment used was not a strong enough inducing treatment

4.6. The cloned *ssb* and *psiB* genes are not inducible by UV-irradiation and mitomycin C treatment.

Following the observation that *ssb* and *psiB* on ColIb and ColIb*drd-1* are inducible by UV-irradiation and mitomycin C treatment, a similar experiment was performed using the cloned *ssb* 'ON' and *psiB* 'ON' fusions,

Table 4.3. Effect of SOS inducing treatments on expression of ColIb *ssb*, *psiB* and *sog* genes carrying the *lacZ*-Km^r promoter probe in *recA*$^+$ cells.

	β-galactosidase in MC4100*			
Plasmid	No treatment	+UV	+Mit C	+ Nal
a) ColIb and ColIb*drd-1* derivatives				
pAL8	0	23	8	0
pAL6	16	137	108	11
pEP5	0	15	9	0
pEP3	33	141	113	25
pAL11	297	249	267	239
pAL18	1671	1436	1339	NT
b) Subcloned *ssb* and *psiB* 'ON' fusions.				
pAL4	104	113	99	NT
pEP1	498	434	477	NT

* Values are units mg^{-1} protein measured in MC4100 (*recA*$^+$) and are the means of at least three experiments. Treatments were: UV irradiation (10 J m$^{-2)}$, mitomycin C (1 μg/ml) and nalidixic acid (40 μg/ml). β-galactosidase was determined after a 1 h incubation at 37°C. NT, not tested.

pAL4 and pEP1 respectively, to determine if the *ssb* and *psiB* genes on these plasmids are also inducible. MC4100 (pAL4) and MC4100 (pEP1) strains were treated with UV-irradiation and mitomycin C in an identical manner to that described above and tested for β-galactosidase production. Table 4.3b shows that in contrast to the *ssb* and *psiB* genes on ColIb and ColIb*drd-1*, expression of the cloned *ssb* and *psiB* genes is not affected by the SOS inducing treatments.

4.7. Effect of UV-irradiation and mitomycin C on expression of *ssb* and *psiB* in *recA*$^-$ and *lexA*(Ind$^-$) strains.

In order to test whether *ssb* and *psiB* on ColIb are induced by the SOS response, it was necessary to determine if the effect was *recA*-dependent and whether it occurred in a *lexA* (Ind$^-$) strain specifying a noncleavable LexA protein. It was expected that if these genes were members of the SOS regulon, there would be no increased expression of *ssb* and *psiB* in a *recA*$^-$ or *lexA* (Ind$^-$) strain. Three host strains were used for these tests, SE5000 (*recA*56), AB2463 (*recA*13), and DM49 (*lexA*3=*lexA*(Ind$^-$). The ColIb*drd-1 ssb* 'ON' fusion, pAL6, and the ColIb*drd-1 psiB* 'ON' fusion, pEP3, were tested in SE5000, AB2463, and DM49 for inducibility following UV-irradiation and mitomycin C treatment.

Table 4.4 shows that the DNA damage inducibility of *ssb* and *psiB* is *recA* independent and occurred in a *lexA* (Ind$^-$) strain. These results suggest that the induction is independent of the SOS regulon. Expression of *ssb* and *psiB* had been shown to be non-inducible by the addition of nalidixic acid, which is a well characterised SOS inducing treatment. Therefore, *ssb* and *psiB* of ColIb and ColIb*drd-1* are damage inducible but the mechanism of induction appears to be different from that of the conventional SOS genes.

Table 4.4 Effect of UV irradiation on β–galactosidase specified by ColIb *psiB* (pEP3) and *ssb* (pAL6) mutants carrying the *lacZ*-Km^r promoter probe in *recA*$^-$ and *lexA*(Ind$^-$) cells.

	β-galactosidase in strain*					
Plasmid	AB2463 (*recA*13)		SE5000 (*recA*56)		DM49 (*lexA*3)	
	-UV	+UV	-UV	+UV	-UV	+UV
pAL6	16	64	15	63	11	34
pEP3	22	103	26	135	29	162

* Values are units mg^{-1} protein and are the means of at least three experiments.
For +UV, strains were irradiated with 10 J m^{-2}. β-galactosidase was determined after a 1 h incubation.

4.8. DNA damage inducibility of *ssb* and *psiB* expression is independent of the heat shock response.

The heat shock response of *E.coli* is inducible by UV-irradiation or mitomycin C treatment with expression requiring the action of the *htpR* gene product (Krueger and Walker, 1984). In order to determine whether the inducibility of *ssb* and *psiB* is via the heat shock response, a series of preliminary experiments were performed. Plasmids pAL6 (ColIb*drd-1 ssb* 'ON') and pEP3 (ColIb*drd-1 psiB* 'ON') were transferred into *E. coli* derivatives LC133 and LC137. LC133 is *htpR*$^+$, whereas LC137 is *htpR*. Thus the heat shock response should be inducible in LC133 but not in LC137. LC137 but not LC133 will grow at 42°C. These strains containing the ColIb*drd-1* fusion derivatives were subjected to the UV-irradiation treatment as described above except that growth was at 30°C. β-galactosidase activity was measured after a one hour incubation. Table 4.5 contains the results of such experiments and shows that expression of the *ssb* and *psiB* genes is induced by UV irradiation in both strains. This result indicates that the DNA damage inducibility is independent of the heat shock response as well as the SOS response.

In order to confirm heat shock independence, actively growing MC4100 cells containing pAL6 or pEP3 were treated to a temperature shift (28°C to 42°C) and β-galactosidase accumulation was measured at various times after the shift to determine if there was induction of *ssb* and *psiB*. There was no increased expression of the *ssb* and *psiB* fusion genes following the change of incubation temperature (data not shown). It is reported that the maximum induction of the heat shock genes should occur 5-10 min following the temperature shift (Krueger and Walker, 1984).

Table 4.5. Effect of UV irradiation on β-galactosidase specified by ColIb*drd-1 ssb* (pAL6) and *psiB* (pEP3) mutants carrying the *lacZ*-Km^r promoter probe in *htpR* and *htpR*$^+$ cells..

	β-galactosidase in strain*			
Plasmid	LC133 (*htpR*+)		LC137 (*htpR*)	
	- UV	+ UV	- UV	+ UV
None	8	7	11	10
pAL6	19	77	16	80
pEP3	29	112	34	109

* Values are units mg^{-1} protein and are the means of at least three experiments. For + UV, strains were irradiated with 10 J m^{-2}.
β-galactosidase was determined after a 1 hr incubation at 30°C.

4.9. Discussion.

The work outlined in this chapter concerns the regulation of expression of ColIb leading region genes, *ssb* and *psiB*. and contrasts it with that of *sog*, a representative transfer gene.

Using the *ssb-*, *psiB-* and *sog-lacZ* transcriptional fusions created in ColIb and ColIb*drd-1*, it was possible to study the expression of these genes under a variety of conditions. The first consideration was to determine whether *ssb* and *psiB* are coordinately regulated with the transfer genes. The precise nature of the ColIb *drd-1* mutation, which allows constitutive expression of the *tra* genes, is unknown. The transfer genes of plasmid F are naturally derepressed due to the presence of an insertion element in the *finO* gene. The product of *finO* normally acts in combination with the *finP* product to repress production of TraJ, the positive regulator required for transcription of the major F transfer operon (Willetts and Skurray, 1987). By analogy with plasmid F, it is likely that the ColIb *drd-1* mutation prevents (or reduces) synthesis of a repressor of the transfer genes.

Through the use of the promoterless *lacZ* fusions to the *ssb* and *psiB* genes on ColIb and ColIb*drd-1*, it was found that expression of both genes is increased when the plasmid transfer system is derepressed. However, the level of expression is much lower than that of *sog*. No β-galactosidase was detectable from the *ssb-* and *psiB-lacZ* fusions on ColIb (wild type). The *ssb-lacZ* and *psiB-lacZ* derivatives of ColIb*drd-1* specified a low level of activity, which was approximately 80-fold less than that from ColIb*drd-1 sog-lacZ*. Thus, it appears that *ssb* and *psiB* do show coordinate regulation with the *tra* genes even though transcription is low. As *sog* was the only transfer gene used in this study, it is not clear whether this gene is expressed at a comparatively high or low level relative to other *tra* genes.

These results are in agreement with genetic evidence concerning the suppression of the temperature sensitivity of an *E. coli ssb-1* mutant by

ColIb *ssb*, the level of suppression being greater when the ColIb plasmid carries a *drd* mutation (Howland *et al.*, 1989). The presence of ColIb*drd-1* almost totally suppresses the temperature sensitivity of the *ssb-1* mutation, even though the level of plasmid *ssb* expression on the *drd* plasmid appeared to be low. Therefore only a low level of plasmid SSB protein is required for suppression of the *ssb-1* mutation. Observations by Howland *et al.* (1989) showed that suppression of the UV sensitivity of *ssb-1* cells by ColIb was independent of the fertility inhibition system. This is surprising as my results indicate that there is no *ssb* expression from the wild type ColIb plasmid. It had been postulated that ColIb *ssb* is expressed at two levels, a low basal level and a higher level coordinately expressed with the transfer functions (Howland *et al.*, 1989). This apparently anomalous result can be explained by the finding that UV irradiation induces expression of the ColIb *ssb* gene. The level of ColIb *ssb* expression in UV irradiated cells is equivalent to that of ColIb*drd-1 ssb* in unirradiated cells. Under the latter conditions, presence of the plasmid can suppress the temperature sensitivity of the bacterial *ssb-1* mutation and so it is possible that the UV induced level of SSB on ColIb although low, is sufficient to suppress the UV sensitivity conferred on the cells by the bacterial *ssb-1* mutation.

Both the ColIb *ssb* and *psiB* genes are inducible by treatment with UV-irradiation or mitomycin C. Expression of the *sog* gene was not affected by such agents and so the induction does not appear to be a general feature of all ColIb genes. This rules out the possibility that the effect is due to an increase in plasmid copy number. The subcloned *ssb-* and *psiB-lacZ* fusions (pAL4 and pEP1 respectively) were tested for DNA damage inducibility and none was observed. The level of expression from the subclones under normal conditions is much higher than from the ColIb plasmids. This may be due to either a copy number effect or to absence of a repressor or repressor binding site. The latter possibility would provide an explanation

for the noninducibility of the subcloned *ssb* and *psiB* genes, since they would already be expressed maximally.

The damage inducibility of *ssb* and *psiB* on ColIb*drd-1* was shown to be *recA/lexA* independent and did not occur following exposure to nalidixic acid. These results would indicate that the induction is independent of the SOS system. It is possible that introduction of lesions into the DNA caused the induction as other methods of inducing the SOS response had no effect. Additionally, no LexA boxes were found upstream of the genes, although the sequence data is not complete in these regions. Induction of *ssb* and *psiB* was shown to be independent of the heat shock response, which is also inducible by UV-irradiation, mitomycin C treatment (Krueger and Walker, 1984). This result is not unexpected since the heat shock response is also induced by nalidixic acid treatment. Therefore the mechanism of the induction is unclear and it would appear to involve a novel system. It is possible that these genes are part of a different inducible network of genes controlled in a similar way to the *recA/lexA* system. However, it is surprising that if this is the case, no other *recA/lexA* independent inducible genes have been identified in the many studies of DNA damage inducible genes. An alternative explanation is that the DNA damage caused by mitomycin C and UV-irradiation may result in a conformational change, which affects gene expression directly or alters repressor binding. It is interesting that evidence suggests that the chromosomal *ssb* gene is SOS inducible whereas plasmid *ssb* induction appears to be SOS independent.

Thus, in established plasmid containing cells, expression of *ssb* and *psiB* is increased when the plasmid transfer system is derepressed or the host cell is exposed to DNA-damaging agents. The regulatory elements controlling expression of these genes is therefore likely to be complicated as here we have two distinct observations.

The insert in the *ssb* gene of pAL12 which results in the production of a Psi$^+$ phenotype was shown to produce approximately ten-fold increased in expression of *psiB*. The increased expression is unaffected by the presence of the intact *ssb* gene, indicating that the effect is *cis*-acting and not due to the truncated SSB protein. The mechanism that results in the increased expression of *psiB* is unclear but it is possible there is transcriptional read-out from the genes inserted in *ssb*. The antibiotic resistance genes within the *ssb* gene of pAL12 and pAL11 are in the same transcriptional orientation as the *ssb* gene itself and the direction of transcription of *ssb* and *psiB* is the same. One implication of this result is that there is no transcriptional terminator between *ssb* and *psiB*, although results in chapter 2 suggest that there is a promoter in this region. Plasmid pLG288 (C. Howland, 1989; Howland *et al.*, 1989), like pAL12, has the ability to confer a Psi$^+$ phenotype on the host cell. pLG288 has a Tn*903* derived Km^r cassette in the *ssb* gene of a ColIb*drd-1* derived plasmid, pLG273, with the cassette inserted in the same transcriptional orientation as the *ssb* gene. Presumably in this case the insert within *ssb* is increasing expression of *psiB* by transcriptional read-out from the antibiotic resistance gene, as appears to be the case for pAL12. Thus, the Psi$^+$ phenotype conferred by pLG288 and pAL12 may be an artificially created equivalent to that existing in plasmids R100 and R6-5. The constitutive Psi$^+$ phenotype exhibited by these F-like plasmids has been attributed to the presence of a Tn*10* insertion, with the outward reading promoter of the transposon increasing transcription of the downstream *psiB* gene (Dutreix *et al.*, 1988). Plasmids such as F and ColIb, which carry a *psiB* gene but do not have such a transposon insertion, do not naturally display a Psi$^+$ phenotype.

Chapter 5.

Zygotic induction of *psiB* and *ssb* on ColIb*drd-1*.

5.1. Introduction

The *ssb* and *psiB* genes, being located in the leading region of the plasmid, will be amongst the first genes to enter the recipient cell upon transfer. All *ssb* and *psiB* genes studied thus far are similarly located and it may be important that they are expressed rapidly upon entry. The construction of *lacZ* fusions to *ssb* and *psiB* on ColIb*drd-1* provide an obvious tool for monitoring expression of these genes during a conjugation experiment, as the inserts do not significantly affect the conjugative efficiency of the ColIb*drd-1* derivative (chapter 6). I have already shown that in established plasmid-containing cells expression of *ssb* and *psiB* on ColIb is increased by a *drd* mutation, implying a possible role for the gene products in conjugation. Therefore it was of interest to investigate the effect of transfer on expression of the *ssb* and *psiB* genes.

5.2. Effect of conjugation on expression of ColIb*drd-1 ssb* and *psiB* genes.

Using the ColIb*drd-1 ssb* 'ON' and *psiB* 'ON' fusions, pAL6 and pEP3, expression of the *ssb* and *psiB* genes was monitored during a conjugation experiment. This was performed by taking samples at regular intervals from a 1:1 (vol/vol) mating mixture of plasmid containing MC4100 donor cells and plasmid free MC4100 recipients. MC4100 was used for both parental strains as it carries a chromosomal *lac* deletion and so does not specify any β-galactosidase. A spontaneous nalidixic acid resistant mutant of MC4100 was isolated to use as the recipient strain in these conjugation experiments. This allowed the selection of transconjugants using nalidixic acid and kanamycin to demonstrate that conjugation had occurred and that it was efficient.

The level of β-galactosidase specified by pAL6 and pEP3 was monitored prior to and during a 60 minute conjugation period. Fig 5.1a and 5.1b. shows that accumulation of β-galactosidase specified by the *ssb-lacZ* fusion increased by 11-fold and *psiB-lacZ* by 17-fold shortly after initiation of conjugation. The level of β-galactosidase measured in pAL6 directed conjugations was always approximately four fold less than in pEP3 conjugations. Transconjugant yields showed that the conjugation was efficient. When the recipient cells harboured pLG221 (ColIb*drd-1*) and so expressed entry exclusion preventing homosexual conjugation, no increase was observed (data not shown). It is clear from these results that high level expression of *ssb* and *psiB* is triggered by the act of conjugation. Prior to mixing of parental cultures at t= 0 there is a steady level of β-galactosidase in strains MC4100 (pAL6) and MC4100 (pEP3). At t=0 the parental cultures were mixed and thus the specific activity halves as the recipient strain does not contain any β-galactosidase. The increase in β-galactosidase activity specified by the ColIb*drd-1 psiB-lacZ* fusion is rapid and transient, with the level reaching a peak at approximately 30 minutes and then declining due to the increasing mass of the culture. This point is well illustrated if the Figures for units of β-galactosidase per ml of mating mixture are studied as these values do not take into account the growth of the culture. Values of units per ml of mating mixture increase rapidly after t= 0 but reach a plateau after 30 minutes (Fig. 5.2b.). The accumulation of β-galactosidase specified by the ColIb*drd-1 ssb-lacZ* fusion is less rapid than that for the *psiB-lacZ* fusion and does not display the same levelling off (Fig. 5.1a; Fig. 5.2a.).

These experiments were performed using plasmids that were by default *ssb* or *psiB* mutants and a concern was that the gene products normally control expression of their respective genes by autoregulation. The conjugation experiments were therefore repeated with recombinants carrying the intact *ssb* and *psiB* genes present with the ColIb*drd-1* fusions in

Fig. 5.1. Zygotic induction of (a) *ssb* and (b) *psiB* on ColIb*drd-1*. Values (units mg^{-1} protein) at t = -40 to -2 show specific activity of β-galactosidase in donor strains MC4100 (pAL6) and MC4100 (pEP3), respectively. At t = 0, equal volumes of donor cells and MC4100 recipients were mixed and incubated further and samples taken at the times indicated.

Fig.5.1

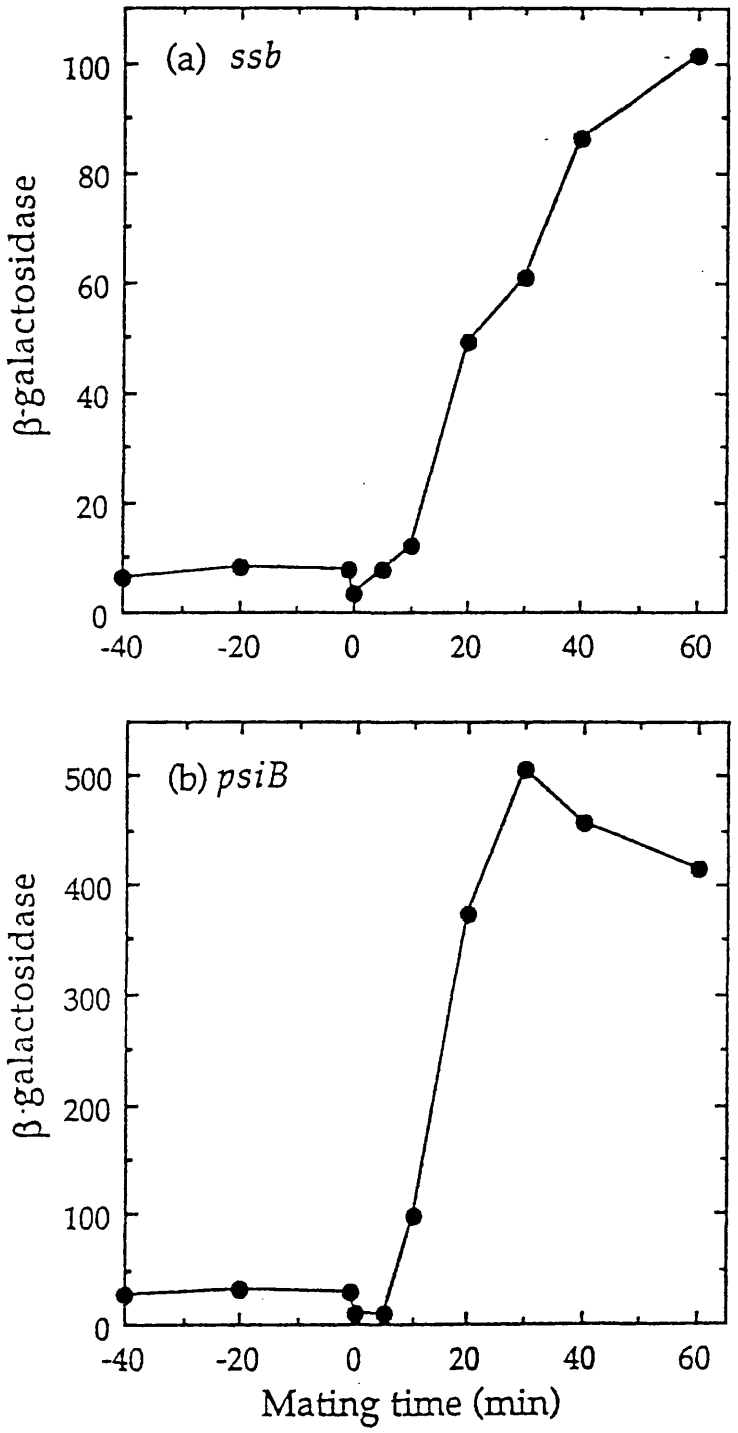

(a) ssb
β-galactosidase
100
80
60
40
20
0
-40
-20
0
20
40
60
(b) psiB
β-galactosidase
500
400
300
200
100
0
-40
-20
0
20
40
60
Mating time (min)

Fig. 5.2. Zygotic induction of (a) *ssb* and (b) *psiB* on ColIb*drd-1*. Values at t = -40 to -2 show units ml^{-1} of β-galactosidase in donor strains MC4100 (pAL6) and MC4100 (pEP3), respectively. At t = 0, equal volumes of donor cells and MC4100 recipients were mixed and incubated further and samples taken at the times indicated. Values are units ml^{-1} mating mixture after t = 0.

Fig.5.2.

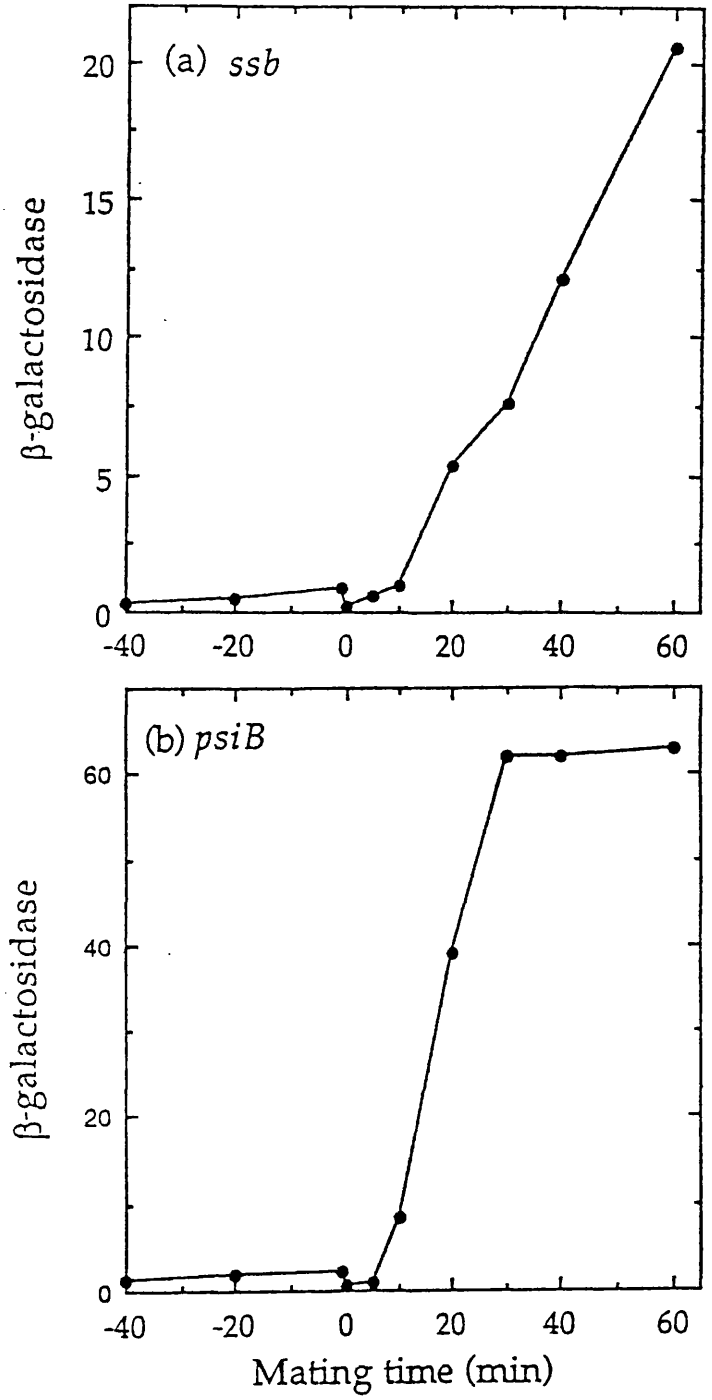
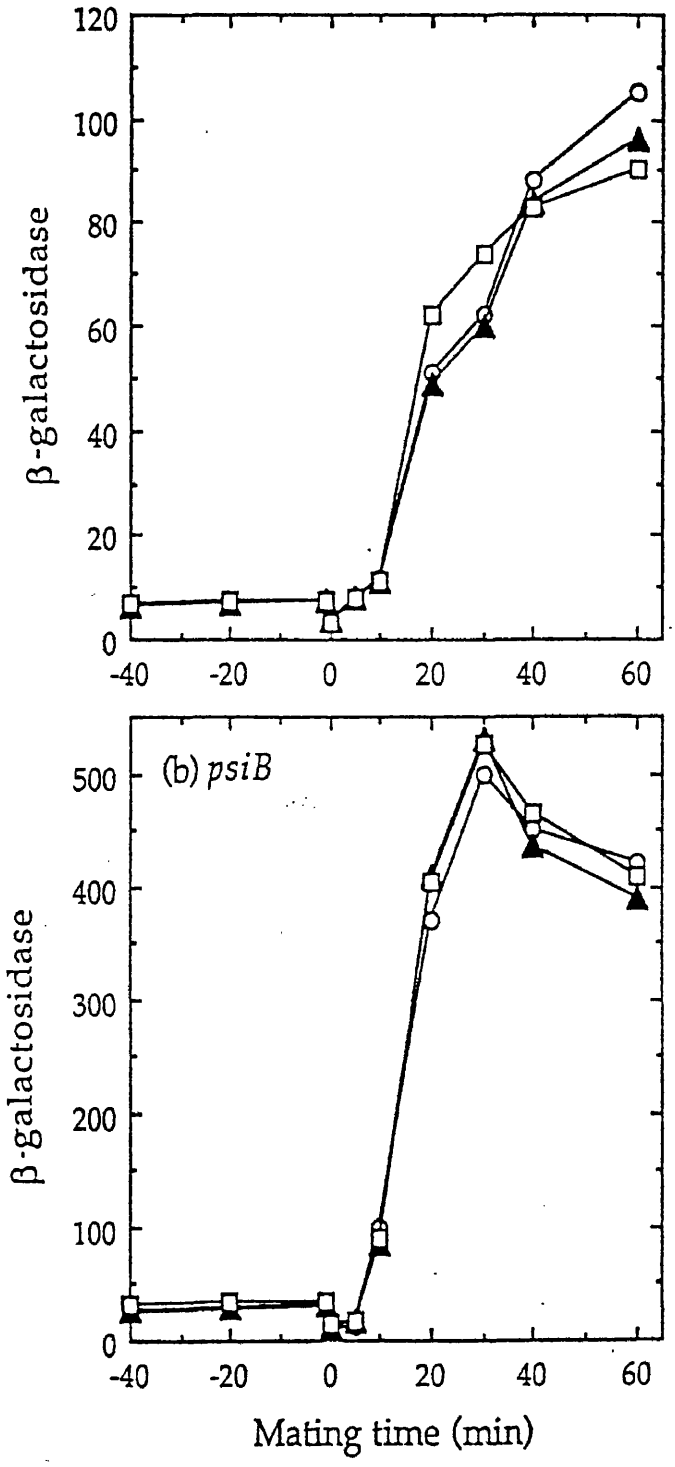

(a) ssb
β-galactosidase
0
5
10
15
20
-40
-20
0
20
40
60
(b) psiB
β-galactosidase
0
20
40
60
-40
-20
0
20
40
60
Mating time (min)

Fig. 5.3. Effect of (a) *ssb*$^+$ and (b) *psiB*$^+$ recombinants, present in donor or recipient strains, on the zygotic induction of *ssb* and *psiB* on ColIb*drd-1*. The results of a control conjugation in which there was no recombinant plasmid present in either parental strain are also shown. Values at t = -40 to -2 show specific activity of β-galactosidase in donor strains (a) MC4100 (pAL6) or MC4100 (pAL6, pLG284) and (b) MC4100 (pEP3) or MC4100 (pEP3, pAL1001). At t = 0, equal volumes of donor and recipient cells were mixed and incubated further and samples taken at the times indicated. Recipients were (a) MC4100 or MC4100 (pAL6) and (b) MC4100 or MC4100 (pAL1001). Symbols are: recombinant plasmid present in the donor strain (filled triangles), recombinant plasmid present in recipient (circles) and no recombinant plasmid present in either strain (squares).

Fig.5.3.


β-galactosidase
120
100
80
60
40
20
0
-40
-20
0
20
40
60
(b) psiB
β-galactosidase
500
400
300
200
100
0
-40
-20
0
20
40
60
Mating time (min)

the donor cells, or in recipient cells. However, the presence, in either donor or recipient population, of pCH4 (*ssb*$^+$) for pAL6 directed conjugations or pAL1001 (*psiB*$^+$) for pEP3 conjugations did not affect the increased levels of β-galactosidase suggesting autoregulation is not involved (Fig. 5.3)

5.3. Localisation of the increase in *ssb* and *psiB* expression

In order to determine whether this increase in *ssb* or *psiB* expression occurs in donor or recipient cells, a series of mating experiments were performed using combinations of MC4100 donors and recipients that were either resistant or sensitive to phage T6. This allows one of the parental strains to be eliminated after the conjugation period (Wilkins *et al.*, 1971). The donors harboured either pAL6 (ColIb*drd-1 ssb* 'ON') or pEP3 (ColIb*drd-1 psiB* 'ON'). A 40 minute conjugation experiment was performed using 1:1 (vol/vol) ratio of donors and recipients. After 40 minutes, the phage sensitive cells were destroyed by lysis-from-without with phage T6 and the amount of β-galactosidase in the resistant strain determined. Table 5.1 shows that the conjugation-dependent increase in *ssb* and *psiB* expression occurs in the recipient cells, as a significant level of β-galactosidase was only detectable when the recipient cells were T6 resistant. The values in Table 5.1 are expressed as units per ml of mating mixture. During the lysis procedure, phage T6 resistant carrier cells are added to aid pelleting of the cells during washing. Hence, it was unsatisfactory to express the values as specific activity (units mg^{-1} protein). The values specified by the *ssb-lacZ* fusions are again lower than those specified by the *psiB-lacZ* fusions.

Table 5.1. Increased *psiB* and *ssb* expression in recipient cells.

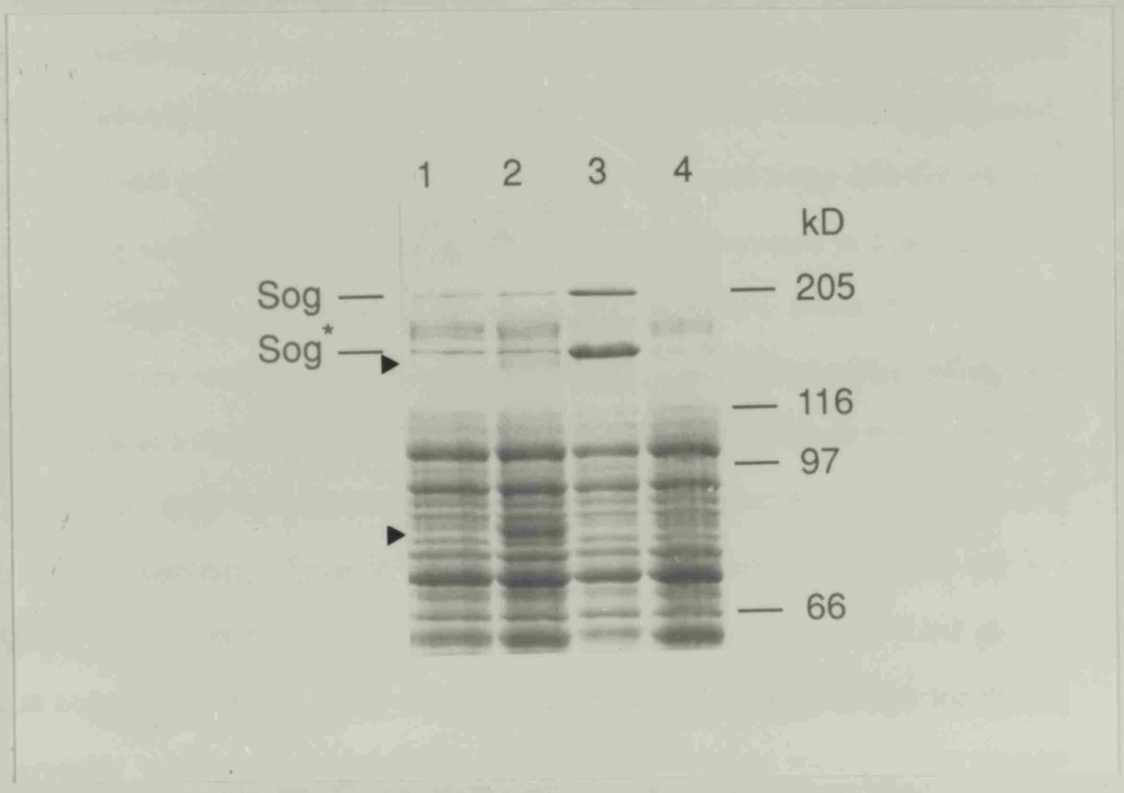
Donor	Recipient	β-galactosidase*
$T6^s$ (pEP3)	$T6^s$	0.89
$T6^r$ (pEP3)	$T6^r$	31.9
$T6^r$ (pEP3)	$T6^s$	1.26
$T6^s$ (pEP3)	$T6^r$	26.7
$T6^s$ (pAL6)	$T6^s$	0.21
$T6^r$ (pAL6)	$T6^r$	8.5
$T6^r$ (pAL6)	$T6^s$	0.35
$T6^s$ (pAL6)	$T6^r$	6.6

* Values are units ml^{-1} mating mixture following a 40 min conjugation and the removal of the $T6^s$ strain by phage T6 lysis procedure. Strains were MC4100 and a $T6^r$ derivative. pEP3 and pAL6 contain *lacZ* fusions to *psiB* and *ssb* on ColIb*drd-1* respectively.

5.4. Expression of *sog* during conjugation.

To investigate whether the zygotic induction observed for *ssb* and *psiB* is a general property of all ColIb transfer genes, I wanted to perform a similar experiment to those described in section 5.2 using the ColIb*drd-1 sog* 'ON' fusion, pAL18. However, this was not possible as pAL18 is highly defective in conjugative transfer and could not be complemented, even through the use of cosmids containing large ColIb fragments (chapter 4). The *lacZ*-Km^r cassette used in the construction of pAL18 may be exerting a strong polarity effect on expression of downstream *tra* genes as the cassette is designed to prevent transcription emanating from its termini. Therefore, a more direct method was used to measure *sog* expression during conjugation. Whole cell extracts were made prior to and following a 40 minute conjugation with pLG221-containing GI65 donors and plasmid free GI65N recipients. pLG221 is a derivative of ColIb*drd-1* with a Tn*5* insertion in the *cib* (Colicin Ib) gene. This insert does not affect the conjugative efficiency of the plasmid. The *sog* gene specifies two polypeptides of apparent molecular weight 210 kD and 160 kD and these were visualised following SDS-PAGE (Rees and Wilkins, 1989). To aid identification of the Sog polypeptides specified by ColIb*drd-1*, a whole cell extract of a strain carrying pBS215-1 was also prepared. pBS215-1 carries the cloned ColIb *sog* gene under the control of a *tac* promoter and will overproduce the Sog polypeptides upon induction with IPTG. Figure 5.4 shows there to be no enhanced synthesis of Sog polypeptides during conjugation (compare lanes 1: GI65 (pLG221) immediately prior to mating and lane 2: mating mixture 40 minutes after mixing of parental cultures). The Sog polypeptides on this gel run at 200 kD and 150 kD which is slightly lower than previously reported. This difference may be attributed to alterations in the gel running conditions. There are bands visible in lane 2 (mating mixture) which are

Fig. 5.4. Quantification of Sog polypeptides synthesized before and after conjugation. Coomasie blue-stained profiles of whole cell extracts. Lane 1, C600 (pLG221) donors; lane 2, mating mixture after 40 min incubation of equal volumes of C600 (pLG221) donors and C600 recipients; lane 4, plasmid-free C600. Lane 3 contains whole cell extract from a strain containing pBS215-1, a plasmid that overexpresses the Sog polypeptides. Bands corresponding to the two Sog polypeptides are indicated as is the positions of two bands that increase in intensity following conjugation (filled triangles).



more visible than the counterparts in lanes 1 or 4 (donors and plasmid-free recipients, respectively) indicating that there may be zygotic induction of other plasmid genes or induction of host genes during conjugation. The positions of two such bands are indicated on Figure 5.4 but their identity is unknown. The zygotic induction observed for *ssb* and *psiB* is clearly not a general property of all plasmid genes.

5.5. Discussion

There is a burst of activity from the *ssb* and *psiB* genes on ColIb*drd-1* shortly after initiation of conjugation. This increase elevates specific activities specified by the *ssb-* and *psiB-lacZ* fusions by at least ten-fold and occurs exclusively in the recipient cells. Thus, even in a ColIb*drd-1* plasmid, the *ssb* and *psiB* genes may be partially repressed and only briefly escape from such repression upon transfer. This repression system is distinct from that controlling the expression of the transfer genes since the zygotic induction occurs with a *drd* plasmid and there was no similar enhanced synthesis of Sog polypeptides. During the analysis of the Sog polypeptides by SDS-PAGE it was apparent other polypeptides increased in amount following conjugation. Thus it is likely that other plasmid genes are subject to zygotic induction upon transfer or that there is induction of host genes, but it is not a general property of all plasmid genes. It would be interesting to investigate which other genes are similarly induced as it may be common to loci in the leading region. For non-essential genes, this could be examined using the *lacZ*-Km^r cassette as for *ssb* and *psiB*.

It appeared to take approximately thirty minutes for repression of the *psiB* gene to be established following transfer. However, this value is inaccurate since the experiment is performed with an unsynchronised population of mating cells. After this time there was no further increase in the accumulation of β-galactosidase specified by the ColIb*drd-1 psiB-lacZ*

fusion. Willetts (1984) proposed that the delay before transfer inhibition is established after entry of F (*finO/finP*$^+$) into R100 (*finO*$^+$/*finP*$^+$)-containing recipient cells is due to transient synthesis of *traJ* product. TraJ allows synthesis of the gene products required for conjugative DNA transfer, even in the presence of the transfer inhibitor. It is suggested that the delay in the transfer inhibitor reaching an inhibitory level allows transient synthesis of TraJ. If expression of the ColIb *ssb* and *psiB* genes is controlled by the action of a repressor then the burst of expression observed from these genes upon transfer may be by a similar mechanism to the transient expression of *traJ*, although clearly the gene products are very different. Thus, in this model the repressor of *psiB* and *ssb* may be equivalent to the FinOP repressor of the F-like plasmids.

The levels of β-galactosidase specified by the *ssb*- and *psiB-lacZ* fusions following conjugative transfer are very different, with *psiB* always more highly expressed. The profiles from the zygotic induction experiments are also distinct (Fig 5.1a and 5.1b). Previous observations had shown that certain inserts in the *ssb* gene can affect the level of transcription of the downstream *psiB* gene, presumably by providing a strong outward reading promoter. This raised the possibility that *ssb* and *psiB* are transcribed as a single polycistronic message, since there did not appear to be a transcriptional terminator located between the two genes. However, the zygotic induction data suggest that it is unlikely that the *ssb* and *psiB* genes are simply transcribed as a single message, as the level of *psiB* expression appears to be higher than *ssb*. Experiments described in Chapter 2 indicated that there is a promoter located in between *psiB* and *ssb*. I propose that both genes have upstream promoters, which are regulated by the same type of system, but a transcript initiated upstream of *ssb* can extend to include *psiB*.

The putative repressor of *ssb* and *psiB* does not seem to be the same as that controlling the transfer genes and so it is difficult to use this model to

explain the results in Chapter 4 showing increased level of expression of *ssb* and *psiB* on ColIb carrying a *drd* mutation and the damage inducibility data. If the repressor is analogous to LexA, and can be inactivated by a DNA damage-dependent mechanism, then this would explain the induction of *ssb* and *psiB* by UV irradiation and mitomycin C treatment (chapter 4). There may be a general increase in transcription of all plasmid genes when there is a *drd* mutation.

Informative future work in this area could involve the identification of the repressor of *ssb* and *psiB* and the repressor of the ColIb *tra* genes. The repressor of the *ssb* and *psiB* genes could be identified using cloned fragments of ColIb in the recipient cell and searching for those which will prevent zygotic induction following conjugation. It would then be interesting to investigate whether such a repressor was cleaved or modified by DNA damaging agents.

The work described in this chapter would have been greatly facilitated by knowledge of the regulatory elements controlling expression of the ColIb transfer genes. The construction of the ColIb *sog* 'ON' and ColIb*drd-1 sog* 'ON' plasmids could be used for the identification of the repressor of the transfer genes. The *drd* plasmid, when present in a strain carrying a chromosomal *lac* deletion, will specify enough β-galactosidase to cause production of blue colonies on media containing X-gal, whereas the wild type plasmid will not and the colonies are white (results not shown). By exploiting this observation to select for an intact fertility inhibition system, it should be possible to transform a strain carrying ColIb*drd-1 sog* 'ON' with cloned fragments from ColIb (wild type) and to identify those which result in the production of white colonies. Presumably such clones would contain DNA encoding the repressor, which is defective in the ColIb*drd-1* plasmid.

Chapter 6.
Physiological function of PsiB.

6.1. Introduction.

Through the use of the ColIb*drd-1 ssb-lacZ* and *psiB-lacZ* fusions it has been demonstrated that there is a burst of expression from these genes in the recipient cell following conjugative transfer. The implication is that SSB and PsiB proteins function in the transconjugant cell, rather than in the primary donor. Studies of the plasmid SSBs have been uninstructive in defining a role for these proteins (Golub and Low, 1986; Howland *et al.*, 1989). A ColIb*drd-1 ssb* mutant showed no defects in terms of transconjugant formation or maintenance stability. It was postulated that PsiB functions to prevent triggering of the SOS response by conjugative transfer of single-stranded DNA (Bagdasarian *et al.*, 1986). Circumstantial evidence of a role for PsiB in conjugation is the detection of sequences homologous to the R6-5 *psiB* gene on diverse conjugative plasmids belonging to nine different incompatibility groups. The same set of plasmids also carry related *ssb* genes (Golub *et al.*, 1988).

This chapter concerns the possible role of PsiB. By default, through the creation of the ColIb*drd-1 psiB::lacZ* fusion, a *psiB* mutant had been made. For tests of the properties of a ColIb*drd-1 psiB* mutant, the *psiB* 'NO' fusion, pEP4, was used. Since the *lacZ*-Km^r fragment in this construct had been introduced at a *Pst*I site, 50 bp into the coding region of *psiB*, it is unlikely that a biologically active polypeptide is produced from this short 5' region. No β-galactosidase is determined in the 'NO' orientation as it is the Km^r gene on the fragment, rather than *lacZ* that is in the same transcriptional orientation as *psiB* (chapter 4, section 4.2). Some of the tests used involved *sfiA::lacZ* expression studies and so it was essential to use the 'NO' mutant, pEP4.

6.2. Conjugative properties of a ColIb*drd-1 psiB* mutant, pEP4.

6.2.1. Effect of a ColIb *psiB* mutation on transconjugant formation.

The conjugative efficiency of the ColIb*drd-1 psiB*::NO mutant, pEP4, was assessed in liquid matings of *E. coli* K-12 donors and recipients. If the PsiB protein has a major role in the conjugative process, it should be detectable genetically. Thus, the transconjugant yield in a pEP4-directed mating was compared to that using pLG221-containing donors. The parental plasmid of pEP4 was ColIb*drd-1* which has no selectable marker and so could not be used in this type of quantitative experiment. pLG221 is a derivative of ColIb*drd-1* with a Tn*5* insertion within the *cib* (Colicin Ib) gene (Boulnois, 1981), which does not affect conjugative efficiency.

Table 6.1. shows the transconjugant yields at time intervals up to and including 40 minutes. The results clearly show that although pEP4 forms slightly fewer transconjugants than pLG221, there is no absolute requirement for an intact *psiB* gene for conjugation.

6.2.2. Effect of conjugative transfer of the ColIb *psiB* mutant on expression of an SOS reporter gene in donor and recipient cells.

Bagdasarian *et al.*, (1986) proposed that PsiB prevents SOS induction during transfer of single-stranded DNA. This hypothesis was tested by monitoring expression of an SOS reporter gene in donor and recipient cells during conjugation mediated by pEP4 (ColIb*drd-1 psiB*::NO). The host strain for both donor and recipients was GC4415, which is *recA*$^+$ and carries a *sfiA::lacZ* fusion such that SOS functions can be followed by monitoring β-galactosidase levels. The SOS inducibility of the *sfiA::lacZ* fusion in this strain was demonstrated in Chapter 4. Treatment of the strain with UV-irradiation or mitomycin C resulted in approximately a 30-fold increase in β-galactosidase accumulation. The *sfiA* gene has a high basal level of

Table 6.1. Transconjugant formation by a *psiB* mutant of ColIb*drd-1*.

Time (min)	pLG221 (*psiB*$^+$)	pEP4 (*psiB*)	*psiB* / *psiB*$^+$
0	$< 10^2$	4.0×10^2	
5	6.9×10^6	6.1×10^6	0.88
40	1.9×10^8	1.4×10^8	0.73

Donor strains were MC4100 carrying pLG221 or pEP4. C600N was the recipient. Bacteria were grown in nutrient broth. Values show the number of Km^{r}Nal^r transconjugants per ml of mating mixture obtained after the indicated time of conjugation.

expression and is believed to be relatively easily induced by such DNA damaging agents. Hence, it is a suitable system for following the potential triggering of the SOS response during conjugation. As a control, *sfiA::lacZ* expression was also monitored during conjugation directed by pLG221. As this plasmid has an intact *psiB* gene, it was expected that there would be no increase in *sfiA* expression using pLG221-containing donor cells.

A spontaneous, nalidixic acid-resistant isolate of GC4415 (GC4415N) was identified for use as the recipient strain. This allows selection of transconjugants to confirm the conjugative efficiency. The GC4415N isolate was tested for DNA damage inducibility of the *sfiA::lacZ* fusion to ensure that it was identical to that of the parental GC4415 strain (data not shown).

Plasmid-containing GC4415 donor cells were mixed with plasmid-free GC4415N recipients to give a total volume of 6 ml. The donor: recipient ratios were 1:1 and 1:9 (vol/vol). The ratio of 1:9 was used to allow epidemic spread of the plasmid and therefore provide a population of actively conjugating cells. Samples were taken at 30 minute time intervals up to 120 minutes and assayed for β-galactosidase activity. Figure 6.1a and 6.1b shows that there is an increase in *sfiA::lacZ* expression during conjugation directed by pEP4. When the *psiB* gene is intact, as in the pLG221-directed conjugation, there was no detectable increase in β-galactosidase activity during the 120 minutes In fact there was a consistent slight reduction of activity in these matings. The effect of disrupting the *psiB* gene was more evident when the ratio of 1 donor: 9 recipient was used. This is likely to be due to the spread of the plasmid throughout the recipient cell population and thus amplifying the effect. It was apparent that GC4415 harbouring a ColIb plasmid determines a higher background level of *sfiA::lacZ* expression than the plasmid-free strain. Transconjugant yields showed that conjugation was efficient in all the tests and that there was only very slightly fewer transconjugants formed in the pEP4 matings (data not shown).

Fig. 6.1. Expression of *sfiA::lacZ* in conjugating cells.

At t = 0, GC4415 harbouring pLG221 [open circles] or the ColIb*drd-1 psiB* mutant, pEP4 [closed circles], was mixed with GC4415N recipients in a ratio of (**a**) 1:1 and (**b**) 1:9 (vol/vol) ratio and incubated at 37°C. Values are units mg^{-1} protein.

Fig.6.1.

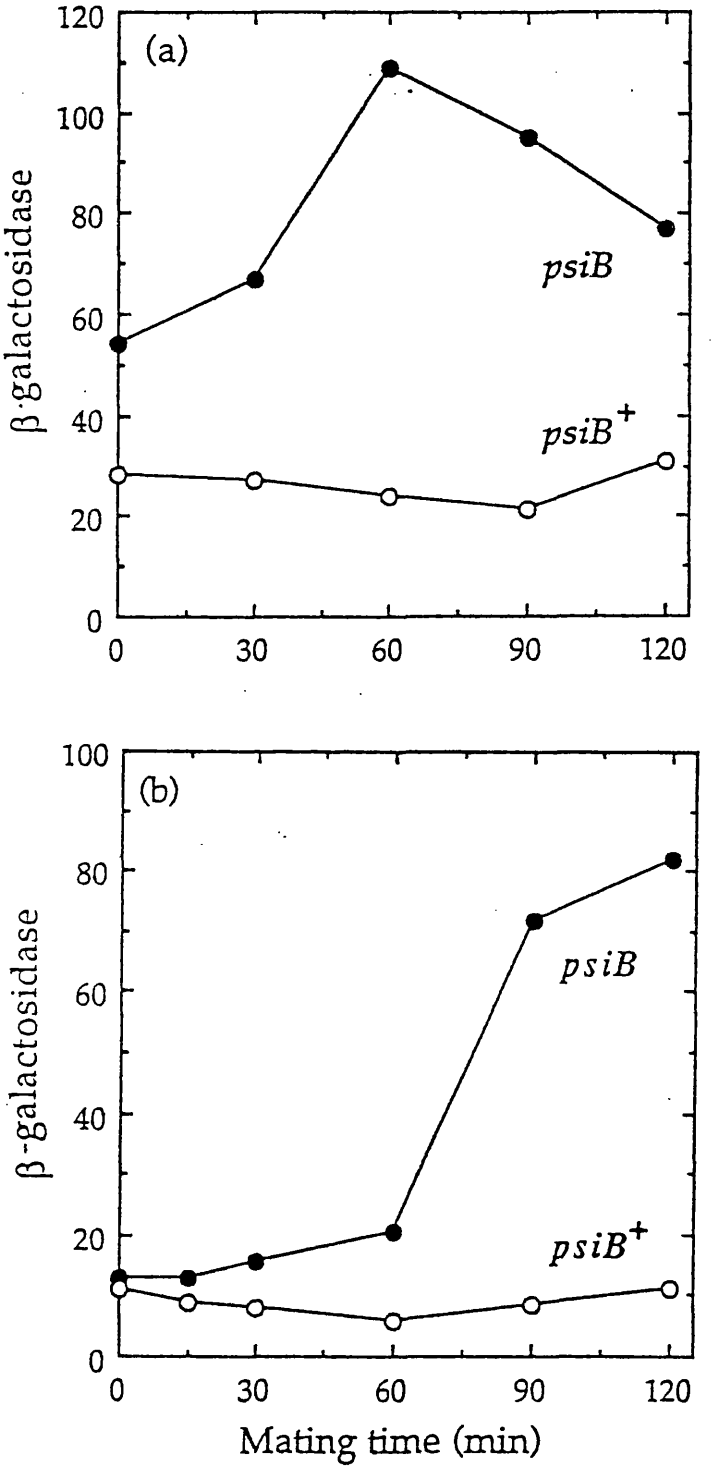

(a)
β-galactosidase
120
100
80
60
40
20
0
0
30
60
90
120
psiB
psiB+
(b)
β-galactosidase
100
80
60
40
20
0
0
30
60
90
120
Mating time (min)
psiB
psiB+

Fig.6.2. Effect of the presence of pAL1001 on expression of *sfiA::lacZ* in conjugating cells.

At t = 0, GC4415 harbouring the ColIb*drd-1 psiB* mutant, pEP4, was mixed with GC4415N recipients in a 1:9 (vol/vol) ratio and incubated at 37°C. The *psiB*$^+$ recombinant plasmid, pAL1001, was present in either donor (open circles) or recipient cells (closed circles). Values are units mg^{-1} protein.

Fig.6.2.

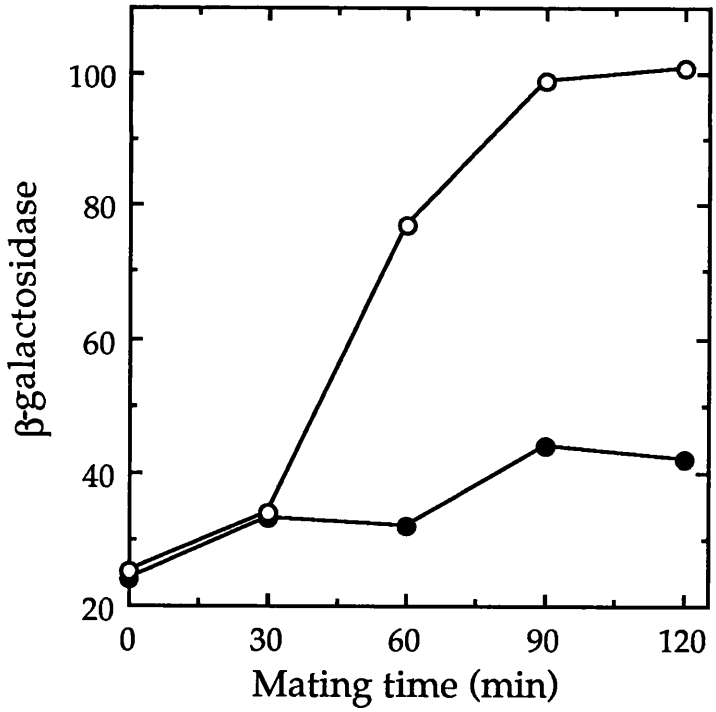


Figure 6.2. shows that presence of pAL1001, which carries the subcloned *psiB* gene, in the recipient cells prevented the increase in *sfiA::lacZ* expression in the pEP4-directed conjugation experiment. When pAL1001 was present in the donor cells, the increase was still observed, but it should be noted that a donor:recipient ratio of 1:9 was used for this experiment and so epidemic spread of the plasmid is expected. As the *psiB* gene is at the distal end of the ColIb fragment in pAL1001 it would indicate that it is presence of the *psiB* gene that prevents SOS induction during conjugation and not some other gene which might be affected by polarity effects conferred by the insertion.

Using a donor:recipient ratio of 1:9, a six-fold increase in *sfiA::lacZ* expression was detected during conjugation when the *psiB* gene is disrupted. This level of SOS induction is lower than that achieved in plasmid-free GC4415 following UV-irradiation or mitomycin C treatment, indicating that the SOS inducing signal is less significant than that generated by conventional DNA damaging agents used in laboratory experiments.

6.3. Discussion

The ColIb *psiB* gene is inessential for conjugation of *E.coli* K-12. In tests of conjugative efficiency, the ColIb*drd-1 psiB* mutant, pEP4, formed only slightly fewer transconjugants than pLG221. This result was not unexpected as it was shown by earlier work that ColIb *ssb* is also inessential for conjugation (Howland *et al.*, 1989). The same applies to the corresponding genes on plasmid F, as shown by the conjugative properties of a plasmid deleted of the leading region (Loh and Skurray, 1989). Thus, it appears that the genes play an auxiliary role and that there is not an absolute requirement for the gene products.

A six-fold increase in the expression of an SOS reporter gene (*sfiA*) was detected in cells following conjugation mediated by pEP4. This result is

consistent with the hypothesis that PsiB acts to prevent triggering of the SOS response during conjugation (Bagdasarian *et al.*, 1986). Carriage of the *psiB* gene on ColIb*drd-1* prevents such SOS induction during transfer, as does the presence of the subcloned *psiB* gene in the recipient cells. Thus the SOS inducing signal is likely to be generated in the recipient cell. Zygotic induction of *psiB*, described in chapter 5, agrees with this conclusion. The slight lowering of expression of *sfiA::lacZ* in pLG221-directed conjugations may reflect zygotic induction of *psiB*. The level of *psiB* expression by zygotic induction is known to be greater than that required for a detectable Psi^+ phenotype.

The level of SOS induction observed is lower than that achieved by UV-irradiation (10 J/m^{-1}) or mitomycin C treatment (1 μg/ml). The latter causes greater than a 20-fold induction of *sfiA::lacZ* after 60 minutes. The *sfiA* gene is reported to be induced relatively easily. Hence, it is possible that the triggering of SOS response observed here may not have been detected if a different reporter gene had been chosen. However, it is unlikely that a cell would ever be exposed to such severe DNA damaging conditions as those imposed by the chemical or UV treatments used in the laboratory. It may be that the subtle effects observed during conjugation when *psiB* is inactive are important.

Chapter 7.
General discussion.

Plasmid ColIb carries a *psiB* locus with 84% identity at the nucleotide level to the *psiB* gene of F. Moreover, the genes map in the same relative position in the leading region of their respective plasmids, downstream of an *ssb* gene. Conservation of this segment of F and ColIb is emphasized by the similar size (2.5 kb) of the region between *psiB* and *ssb* on the two plasmids. The indication is that this whole module is conserved as a unit. Nucleotide sequence similarities extend beyond *psiB*, implying that ColIb carries a *psiA* gene, which on F is located immediately downstream of *psiB* (Loh *et al.*, 1990). The function of *psiA* is unknown, since *psiB* alone is sufficient for Psi function.

The observation that ColIb carries a *psiB* gene is not surprising, as all known plasmids that carry homologous *ssb* genes also carry *psiB*. Plasmids from incompatibility groups FI, FII, FVI, I1, B/O, K, SI and SII belong to a set of plasmids which carry sequences showing similarity with the leading region of plasmid F (Golub and Low, 1985; 1986b). Plasmids F, R100 and R1 are known to carry homologous plasmid maintenance loci in their leading regions, located downstream of *psiA* (Gerdes *et al.*, 1986; Loh *et al.*, 1988). The maintenance locus on F is termed *flm* (*F leading maintenance*), which is also known as *hok /sok* . The *hok* product is a membrane-associated lethal protein (Gerdes *et al.*, 1986). The *sok* locus encodes a non-translated complementary RNA which binds to the *hok* RNA to suppress expression of the lethal Hok polypeptide. The *hok* RNA, which is more stable than the *sok* RNA (Loh *et al.*, 1988; Gerdes *et al.*, 1988), is retained for a longer period following plasmid loss. Thus death of plasmid-free segregants results from the eventual translation of the more stable *hok* RNA in cells which have lost the *hok/sok* locus (Gerdes *et al.*, 1988; 1990). It was thought that all

plasmids that carry *ssb* and *psiB* genes would also have homologous maintenance loci. However, using part of the R1 *hok* gene as a probe, no such homologous region was detected on ColIb (see Appendix I). This result is in agreement with data collected from Northern hybridization experiments, which show large amounts of the unusually stable *sok* RNA is specified by F and R100 plasmids but not by bacteria harbouring IncI1 plasmids R64 or R64*drd-1* (Golub and Panzer, 1988). The sequence of the locus on I1 plasmids may have diverged, such that it would not be identified by hybridization. However, evidence suggests that the extent of homology between the leading regions of I1 and F-like plasmids does not include *hok/sok*. The distance between *psiB* and *oriT* on ColIb (7.5 kb) is greater than on F (3.8 kb) indicating that the *oriT* proximal portion of the leading region of these two plasmids may not be homologous.

That there is some homology between the I1 and F-like plasmids is interesting as members of these incompatibility groups are very different, as judged by their conjugation systems (Rees *et al.*, 1987). The I1 system has an unusual genetic complexity and appears to be derived from two ancestrally distinct lines. Studies on the IncI1 plasmids ColIb and R64, which determine virtually identical conjugation systems, have indicated that there are similarities between the IncI1 Tra2/Tra3 region and the Tra system of IncP plasmids (as detailed in Chapter 1). The Tra2 region of IncI1 plasmids determines a rigid pilus that is essential for conjugation and is morphologically similar to that of IncP and IncM plasmids (Bradley, 1983; 1984). The Tra2 region also specifies a DNA primase encoded by the *sog* gene (Rees *et al.*, 1987). The primase has a functionally similar role in conjugation to the product of the *traC* (*pri*) gene of RP4. However, despite sharing certain structural features, including a conserved primase motif, the two genes do appear to be unrelated (Rees and Wilkins, 1990). The IncI1 *oriT* region contains a consensus sequence present at the nick sites of IncP

oriT loci and the *nik* genes located adjacent to the *oriT* site show organisational and functional similarities to the RP4 (IncP) *traI* and *traJ* genes (Furuya *et al.*, 1991; Pansegrau and Lanka, 1991). Thus, the Tra region of I1 plasmids shows some similarities to an IncP-like system, particularly in the vicinity of the *oriT* site, but on the other side of *oriT* there are homologies with the equivalent region on F-like plasmids. IncP plasmids do not carry *ssb* or *psiB* genes homologous to those of F, although it is possible that they specify functional equivalents. These observations raise questions concerning the evolutionary relationships between these different plasmid groups.

Eight of the nine incompatibility groups of plasmids carrying homologous *ssb* and *psiB* genes have replicons related to the RepFIC family. The converse holds for representatives of the ten groups lacking *ssb* and *psiB* genes (Golub *et al.*, 1988; Couturier *et al.*, 1988). Using part of the RepFIC replicon from an FI plasmid as a probe in a Southern hybridization experiment, I showed that the Rep region of ColIb, mapped by genetic methods (Rees *et al.*, 1987), is phylogenetically related to the RepFIC replicon (appendix II). The RepI1 and RepFIC replicons occupy similar positions, close to the Tra regions of representative plasmids. These observations indicate that there is an ancestral relationship between the plasmid groups that carry *ssb* and *psiB* genes, even though they specify quite distinct conjugation systems. The RepI1/RepFIC replicon and leading region could represent part of an ancient plasmid, from which the plasmids of the RepFIC family are derived. The IncI1 plasmids may have gained the Tra2/3 region and *oriT* site from an IncP-like plasmid, thus explaining the apparent duality of the IncI1 conjugation system.

The fact that the leading region genes are highly conserved would suggest that they have an important role. They are located on conjugative plasmids, which indicates that this role may be in the conjugative transfer

process. However, the *ssb* and *psiB* genes of ColIb are inessential for conjugation of *E. coli* K-12 (Howland *et al.*, 1989; this work) and the same applies to the counterparts on plasmid F (Loh *et al.*, 1989). It is possible that the genes function is strains other than *E. coli* K-12 as these plasmids can be maintained in other enterobacteria. All experiments carried out thus far on *ssb* and *psiB* genes have been performed *E. coli* K-12 hosts and it may be informative to repeat the conjugative efficiency experiments using different host strains.

Psi activity was only observed in established strains when *psiB* is carried on a multicopy plasmid or when ColIb carried an insert in *ssb*, as on pLG288 or pAL12. The Psi$^+$ phenotype conferred by pLG288 and pAL12 is shown to be due to insertions in *ssb* enhancing transcription of the *psiB* gene by ten-fold. Plasmids R100 and R6-5 express a Psi$^+$ phenotype constitutively due to the presence of a Tn*10* insertion, which provides a strong promoter for the downstream *psiB* gene. It is evident that for the Psi$^+$ phenotype to be observed, expression of the *psiB* gene must be increased. The Psi tests are performed on *recA* mutant strains that are easily activated to give the SOS response. Therefore, it is not clear whether inhibition of the SOS response is the primary physiological function of *psiB*, since overexpression of the gene is clearly required. To investigate further the role of the leading region genes, a study of the regulatory systems controlling their expression was undertaken.

Through the use of promoterless *lacZ* fusions to the *ssb* and *psiB* genes on ColIb, it was shown that expression of both genes is increased when the plasmid transfer system is derepressed or the host cell is exposed to an SOS-inducing treatment, such as UV-irradiation or mitomycin C. Damage inducibility of these genes appeared to be *recA/lexA* independent and did not occur following nalidixic acid treatment, These findings suggest that *psiB* and *ssb* are not members of the SOS regulon. The mechanism of

induction is unclear, although preliminary experiments indicate that it is also independent of the heat shock response. The level of *psiB* expression on ColIb induced by DNA damage is lower than the minimal known value required for a detectable Psi$^+$ phenotype. Therefore it was not clear whether the induced level of PsiB would inhibit SOS functions. However, induction of the SOS response by UV irradiation or mitomycin C treatment is possible in strains carrying ColIb plasmids (Howland, 1989) and so the induced level of *psiB* expression on ColIb is not sufficient to confer a Psi$^+$ phenotype.

The fact that expression of both *ssb* and *psiB* is increased by a *drd* mutation, which causes breakdown of the fertility inhibition system, suggests that the leading region and transfer genes share some regulatory features. An implication is that *ssb* and *psiB* do function in conjugation but, the level of expression of *ssb* and *psiB* in strains harbouring ColIb*drd-1* is low compared to that of *sog*, a representative transfer gene. Likewise there is little transcriptional activity in the leading region of plasmid F, which is permanently derepressed for transfer (Cram *et al.*, 1984). However, there is a burst of activity from the ColIb*drd-1 ssb* and *psiB* genes shortly after initiation of conjugation. This increase is located in the recipient cells and elevates specific activities by at least ten-fold giving levels of *psiB* expression greater than that required for a detectable Psi$^+$ phenotype. No enhanced synthesis of Sog polypeptides was apparent following conjugative transfer of the *drd* plasmid, so the zygotic induction observed for *ssb* and *psiB* is not a general property of plasmid genes. One explanation for the zygotic induction is that in established strains the *ssb* and *psiB* genes are under the control of a *trans*-acting repressor. Upon transfer there is a delay in this repressor reaching its inhibitory concentration, resulting in a burst of expression localised to the newly infected recipient cell. An alternative explanation is that the expression of these genes is influenced by the topology of the plasmid DNA (Dorman, 1991; Drlica and Snyder, 1978;

Menzel and Gellert, 1987). In a covalently closed, negatively supercoiled molecule, expression of *ssb* and *psiB* may be inhibited. However, in the recipient cell, transcription from the newly duplexed plasmid may initially be from a linear molecule. It is an interesting possibility that the structure of the plasmid may have an effect on the regulation of gene expression. Preliminary experiments have indicated that expression of ColIb*drd-1 ssb* and *psiB* genes is not increased by relaxation of the plasmid structure using a DNA gyrase inhibitor such as coumermycin A_1 (data not shown).

Using an immunological approach, Bagdasarian *et al.* (submitted for publication) have shown that PsiB concentration is also increased in recipients during F-mediated conjugation. The implication is that PsiB and SSB proteins function in the transconjugant cell, rather than in the primary donor. Zygotic induction is an ideal mechanism to allow expression of these genes for a short period immediately following transfer. Plasmid SSB might participate in complementary strand synthesis on the transferred plasmid strand. However, this process is considered to occur concurrently with DNA transfer and physiologically correct transcription of *ssb* in the leading region presumably requires prior synthesis of duplex DNA. An alternative is that plasmid SSBs augment cellular SSB levels to promote establishment of the immigrant plasmid or secondary transfer of the plasmid from the newly formed transconjugant cell.

It was postulated that PsiB has evolved to prevent SOS induction that may be triggered during the transfer of single-stranded DNA, as it is known that regions of single-stranded DNA can act as a potent SOS inducing signal (Bagdasarian *et al.*, 1986). The ColIb plasmid carries SOS inducible genes and it is likely that the same is true for F-like plasmids. The ColIb SOS regulated genes are, *cib* (colicin Ib), *abi* (abortive phage infection) and *imp* (I-group mutation protection). It is an interesting possibility that *psiB* has evolved and has been maintained by diverse plasmids, as a selfish mechanism to

protect against induction of these plasmid genes. If the primary role of PsiB is to prevent triggering of the SOS response, then this may be a way of preventing unnecessary stress for the host cell by induction of plasmid and host SOS genes.

A ColIb*drd-1 psiB* mutant was used to test the hypothesis that *psiB* acts to prevent triggering of the SOS response during conjugation. A six-fold increase in the expression of a chromosomal SOS reporter gene (*sfiA*) was observed in cells following conjugation directed by the ColIb*drd-1 psiB* mutant. Carriage of the *psiB* gene by the ColIb*drd-1* plasmid prevents such SOS induction during conjugation, as did presence of a *psiB*$^+$ recombinant plasmid in the recipient. When the *psiB*$^+$ recombinant plasmid was in the donor cell, SOS induction was still observed. The implication is that transfer of the ColIb*drd-1 psiB* mutant triggers slight SOS induction in the recipient cell.

It is still not clear whether inhibition of the SOS response by transferring DNA is the primary physiological role of the *psiB*, since it is debatable whether more naked single-stranded DNA accumulates in conjugation than in vegetative DNA replication. The transferred strand of the ColIb plasmid is escorted into the recipient cell by multiple copies of the Sog polypeptides. These proteins are thought to coat the transferred strand, with the DNA primase activity associated with the larger polypeptide initiating rapid synthesis of the complementary DNA strand (Rees and Wilkins, 1989). The strand retained in the donor cell is also unlikely to accumulate in single-stranded form because the rate of DNA synthesis by DNA polymerase III is similar to the rate of DNA transport (Willetts and Wilkins, 1984). Therefore, there is doubt as to whether the transfer of single-stranded DNA by conjugation would trigger the SOS response. The hypothesis of Bagdasarian *et al.* (1986) also raises a problem of timing, since presumably expression of *psiB* requires the prior formation of duplex DNA.

Thus, if the SOS inducing signal is the transferred single-stranded plasmid DNA, then the signal would always precede expression of *psiB*. Therefore, the low level of SOS induction observed following the transfer of the ColIb *psiB* mutant may be attributable to another aspect of the process of plasmid transfer.

It has been mentioned that plasmids that carry *ssb* and *psiB* genes have replicons related to RepFIC (also known as RepFIIA). Rapid initiation of replication at the RepFIC/RepFIIA replicon is thought to occur following conjugation giving a transiently elevated plasmid copy number in the new cell (Womble and Round, 1986). Zygotic induction of *ssb* may imply a role for the gene product in this initial replication of the immigrant plasmid. It is possible that the rapid initiation of plasmid replication following conjugation produces more regions of single stranded DNA than are normally present in the cell during vegetative growth and that these regions could potentially act as SOS inducing signals. Since the presence of a *psiB* gene is always associated with this type of replicon, then PsiB could prevent the low level of SOS induction associated with the immigration of a RepFIC/RepFIIA replicon. The SOS induction observed when the ColIb *psiB* gene is inactivated may reflect the replication of the plasmid in the newly infected recipient cell rather than the process of single-stranded DNA transfer. This proposal agrees with the results of the complementation data indicating that the SOS induction is in the recipient cell. Plasmid groups that are unrelated to RepFIC/RepIIA do not carry sequences homologous to *ssb* or *psiB*, but this does not preclude the possibility that they carry functionally analogous genes.

ColIb carries a gene referred to as *pra* (plasmid-mediated restriction alleviation) which operates to alleviate type I restriction systems during conjugation (Tim Read and Brian Wilkins, submitted). The *pra* gene is located downstream of *psiB* and is transcribed in the same direction as *ssb*

and *psiB*. Like *ssb* and *psiB*, expression of *pra* is enhanced by derepression of the I1 conjugation system and complementation studies of a ColIb*drd pra* mutant imply that the gene acts in conjugation following its expression in the newly infected cell. Type I restriction systems initiate rapid degradation of infecting unmodified dsDNA (Dussoix and Arber, 1962). Therefore, there would be a requirement for rapid expression of *pra* in the newly infected recipient cell if the primary role of the gene is to protect the immigrant plasmid from restriction. It may be a general feature of leading region genes that they are subject to zygotic induction.

In conclusion, there is evidence that the ColIb leading region genes *ssb* and *psiB* have an auxiliary role in the initial events following transfer of the plasmid. Presence of *pra* in the leading region is entirely consistent with this interpretation, since the gene acts to prevent restriction of the immigrant double-stranded plasmid by type I restriction enzymes. These type I systems are encoded by enterobacteria, which are the natural hosts of the ColIb plasmid. Plasmid SSB presumably augments cellular SSB levels, which may be depleted by the conjugation process, and carriage of *psiB* is postulated to prevent a low level of SOS induction associated with the transfer of a RepFIC replicon. The relative order of gene transfer during conjugation is similar for all transferable plasmids studies so far, with the *tra* genes entering the recipient cell last. This may reflect a requirement for the *tra* genes to be expressed in the donor cell for as long as possible. Alternatively, there may be a requirement for the early transfer of replication genes necessary for plasmid maintenance. Consistent with this hypothesis, the primary replicon of plasmid F, RepFIA, is located approximately 12 kb from *oriT* and it is therefore transferred early. However, early transfer of the replication genes is not a general property of conjugative plasmids since the replication genes on ColIb (Rees *et al.*, 1987), R100 (Womble and Round, 1988) and RP4 (Thomas and Smith, 1987) are

located close to one end of the respective transfer regions and are consequently some distance from the leading region. The arrangement of genes on ColIb is such that specialised genes such as *imp, abi* and *cib* enter the recipient cell before the replication functions and so the order of entry is intriguing. However, irrespective of the sequence of gene transfer in conjugation, I propose that the leading region contains genes for which there is a selective advantage in having early expression in the recipient cell. The conservation of *ssb* and *psiB* genes and their location in the leading region of quite different plasmids presumably reflects this advantage.

Chapter 8.
Materials and Methods.

8.1. Plasmids and bacterial strains.

Plasmids and *Escherichia coli* K-12 derivatives used, their relevant genotypes and source are described in Table 8.1.1 and 8.1.2. For the purpose of publication some of the plasmids constructed have been allocated different names from those described in the text. The published names are shown in Table 8.1.3.

Bacterial strains C600N and GC4415N are spontaneous nalidixic acid resistant derivatives of C600 and GC4415, respectively. These were isolated by plating 1 ml of an overnight culture of cells, concentrated to 0.1 ml, onto Luria agar plates containing nalidixic acid at 20 μg/ml.

The chloramphenicol acetyltransferase (*cat*) gene was manipulated in a *Hin*dIII flanked cartridge (Pharmacia Biotechnology). The 4.7 kb *lacZ*-Km^r promoter probe was derived from pKOK6 (Kokotek and Lotz, 1989). pBS215-1, kindly provided by Bettina Strack and Erich Lanka, is a pJF119-based recombinant allowing overexpression of ColIb *sog* from an inducible *tac* promoter. pGY7568 is a pACYC184 derivative carrying the *psiB* gene of R6-5 (Bailone *et al.*, 1988).

8.2. Media and radiochemicals.

8.2.1. Media.

Luria Broth:

per litre distilled water add:	Tryptone	10g
	Yeast extract	5g
	NaCl	5g
		pH 7.0

Table 8.1.1. Bacterial strains.

Strain	Genotype	Source or reference
AB2463	*thr-1 leu6 thi proA2 his4 argE3 lacY galK ara14 mtl-1 xyl-5 tsx-33 sup37*	Howard Flanders and Theriot (1966)
BW85	*leu thyA deoB rpsL cir*	Boulnois et al. (1979)
BW86	*dnaG3 thyA deoB rpsL* Δ *(chlA-uvrB) leu cir*	Boulnois and Wilkins (1979)
C600	*thr-1 leu-6 thi-1 supE44 lacY1 fhuA21*	Bachmann (1987)
DM49	*lexA3 proA2 thr-1 leu-6 his4 thi-1 argE3 lacY galK ara14 xyl-5 mtl-1 tsx-33 sup37*	Mount *et al.* (1972)
GC4415	*rec*$^+$ *thr leu pyrD ura*$^-$ *trp*::Mu *his* Δ*lac gal malB rpsL sfiA99*::Mu d(Ap *lacZ*$^+$)	Huismann and D'Ari (1981)
GC4597	*thr leu his argE proA gal rpsL recA441* (λ*cI indI sfiA:lacZ*$^+$)	Huisman and D'Ari (1983)
GY7221	*recA730* Δ*lacZ sfiA211* (λcI *ind1 sfiA::lacZ*$^+$)	Bailone *et al.* (1988)
JC7623	*recB21 recC22 sbcB15 sbcC*	Kushner *et al.* (1971)
JM12	*recA441 thr leu pro his arg gal rpsL tsx*	Cartellazi *et al.* (1972)
KL450	*ssb-1 gyrA*	Kolodkin *et al.* (1983)
NM522	F$^+$ Δ *(lac-pro) thi-1 supE44 hsdR17* (r_k^-, m_k^+)	
MC4100	*rec*$^+$ *araD139* Δ *(argF-lac) rpsL flb5301 ptsF25 deoC1 rbsR relA1 thiA1*	Casadaban (1976)
RU4406	*thi endA hsdR* Tn*1732*	Ubben and Schmitt (1986)
SE5000	MC4100 *recA56*	Silhavy *et al.*, 1984

Table 8.1.2. Plasmids.

Plasmid	Description	Source or Reference
ColIb-P9	IncI1 repressed for transfer	Laboratory stock
ColIb-P9 *drd-1*	IncI1 derepressed for transfer	Laboratory stock
pLG221	ColIb-P9*drd-1 cib*::Tn*5* Km^r	Boulnois (1981)
pLG273	ColIb-P9*drd-1 cib*::Tn*10 ssb*$^+$ Tc^r	Howland *et al.* (1989)
pLG2001	pBR328 Ω (ColIb *ssb*$^+$ *Eco*RI 20.25 kb) Cm^r Tc^r	Merryweather *et al.* (1987)
pLG283	pBR328 Ω (ColIb *ssb*$^+$ *Sal*I 10.1 kb Δ2.35 kb) Cm^r	Howland *et al.* (1989)
pLG284	pACYC184 Ω (ColIb *ssb*$^+$ *Cla*I-*Sal*I 6.3 kb) Cm^r	Howland *et al.* (1989)
pLG286	pLG284 *ssb*::(*aphA-1 Pst*I 1.2 kb) Cm^r Km^r	Howland *et al.* (1989)
pLG288	pLG273 *ssb*::(*aphA-1 Pst*I 1.2 kb) Km^r Cm^r	Howland *et al.* (1989)
pCH12	pLG286 *aphA-1*::*cat* (*Hin*dIII 0.8 kb)	Howland, 1989
pLG215	pBR328 Ω (ColIb *sog*$^+$ *Eco*RI 13 kb)	Merryweather *et al.* (1986)
pKOK6	*Pst*I 4.7 kb *lacZ*-Km^r	Kokotek and Lotz (1989)
pACYC184	Cm^r Tc^r	Chang and Cohen (1978)
pBR328	Ap^r Cm^r Tc^r	Soberon *et al.* (1980)
pUC19	Ap^r	Yanisch-Perron *et al.* (1985)
pBluescript (SK-)	Ap^r	Strategene Cloning Systems
pHG165	Ap^r	Stewart *et al.* (1986)

Table 8.1.3. Plasmids constructed in this work.

Plasmid	Description	Published as
pALS4	pBR328 Ω (pLG288 *ssb*::*aphA-1* *Sal*I 11.2 kb)	pLG2031
pAL1	pBluescript(SK$^-$) Ω (ColIb *Sal*I-*Pst*I 2.0 kb)	pLG2030
pAL1000	pHG165 Ω (ColIb *Sau*3A 2.7 kb)	pLG2032
pAL1001	pHG165 Ω (ColIb *Sau*3A 2.7 kb)	pLG2033
pAL4	pLG284 *ssb*::(4.7 kb *lacZ*-Km^r 'ON')	
pAL5	pLG284 *ssb*::(4.7 kb *lacZ*-Km^r 'NO')	
pAL6	ColIb*drd-1* *ssb*::ON	pLG2042
pAL7	ColIb*drd-1* *ssb*::NO	pLG2043
pAL8	ColIb *ssb*::ON	pLG2044
pAL9	ColIb *ssb*::NO	pLG2045
pEP1	pAL1001 *psiB*::(4.7 kb *lacZ*-Km^r 'ON')	
pEP2	pAL1001 *psiB*::(4.7 kb *lacZ*-Km^r 'ON')	
pEP3	ColIb*drd-1* *psiB*::ON	pLG2038
pEP4	ColIb*drd-1* *psiB*::NO	pLG2039
pEP5	ColIb *psiB*::ON	pLG2040
pAL10	pUC19 Ω (ColIb *Bgl*II 4.7 kb)	
pAL101	pAL10 *sog*::Tn*1732*	
pAL1011	pAL101 *sog*::*Eco*RI	
pAL13	pUC19 Ω (pAL1011 *Eco*RI-*Sal*I 2.3 kb)	pLG2050
pAL14	pAL13 *sog*::(4.7 kb *lacZ*-Km^r 'ON')	
pAL15	pAL13 *sog*::(4.7 kb *lacZ*-Km^r 'NO')	
pAL17	ColIb *sog*::ON	pLG2046
pAL18	ColIb*drd-1* *sog*::ON	pLG2048
pAL19	ColIb*drd-1* *sog*::NO	pLG2041
pAL12	ColIb*drd-1* *ssb*::*aphA-1*::*cat* (*Hin*dIII 0.8 kb)	pLG2036
pAL11	pAL12 *psiB*::ON	pLG2037

ON and NO refer to the orientation of the *lacZ*-Km^r cassette. ON, *lacZ* orientated in the same transcriptional orientation as the target gene, NO, *lacZ* in the opposite orientation.

M9-Glucose Minimal Media:

per litre distilled water add:		
	Minimal Salts	100ml
	20 % w/v glucose	50ml
	5 % CasAmino acids	10ml
	vitamin B1 (1 %)	1ml
	CaMg salts	10ml

Minimal Salts (boil to dissolve)

per litre:		
	Na_2HPO_4	60 g (anhydrous)
	K_2HPO_4	30 g (anhydrous)
	NaCl	5 g
	NH_4Cl	10 g

CaMg salts		
	$CaCl_2$	0.01 M
	$MgSO_4$	0.1 M

Soft Nutrient Agar:

per litre:		
	Oxoid No2 Nutrient Broth	25 g
	Davis agar	5 g

SOC:

per litre:		
	Bacto tryptone	20 g
	Yeast extract	5 g
	1 M NaCl	10 ml
	1 M KCl	2.5 ml

Vitamin B1 (thiamine-HCl) was prepared in distilled water, filter sterilised and stored at 4°C.

Solid Luria or minimal agar was prepared as above with the addition of agar to 1.5 %.

Antibacterial agents.

Antibacterial agents were added to the medium were necessary at the following concentrations: ampicillin (Ap), 100μg ml^{-1}; chloramphenicol (Cm), 25μg ml^{-1}; kanamycin (Km), 50μg ml^{-1}; nalidixic acid (Nal), 25μg ml^{-1}; streptomycin (Sm), 200μg ml^{-1}; and tetracycline (Tc), 7.5μg ml^{-1}, unless otherwise stated.

8.2.2. Radiochemicals.

Radiochemicals were supplied by Amersham International.

[α-^{32}P]dCTP: specific activity was 11.1 TBq $mmol^{-1}$, at a concentration of 3.7 MBq ml^{-1}.

[α-^{35}S]dATP: specific activity was 44 TBq $mmol^{-1}$, at a concentration of 3.7 MBq ml

8.3. Phenotypic characterisation of bacterial strains.

8.3.1. Suppression of *dnaG*.

Suppression of the temperature sensitive *dnaG3* phenotype of host strains carrying ColIb plasmids, was tested by their ability to form colonies at the non-permissive temperature, 40°C. An overnight culture of the test strain was diluted to OD_{600} of 0.35 and then serially diluted to give single colonies on Luria agar. The resulting colonies from overnight growth at 30°C were replica plated onto pairs of plates and one incubated at 30°C and the other at 40°C overnight. Suppression of the temperature sensitivity (colony-forming ability) was measured as the percentage survival of the strain at the non-permissive temperature.

8.3.2. Suppression of *ssb-1*.

Suppression of the temperature sensitive *ssb-1* phenotype of host strains carrying ColIb plasmids was determined by plating dilutions of overnight cultures onto prewarmed Luria agar plates followed by incubation of replicate plates at 30°C and 44°C overnight as described in section 8.3.1..

8.3.3. Sensitivity to bacteriophage.

Sensitivity to bacteriophage was tested qualitatively by cross streaking overnight cultures of the cells across a suspension of the phage which had been dried onto a Luria agar plate. For isolation of phage T6 resistant mutants, the above procedure was used. Resistant colonies were picked, purified to single-colonies and retested for T6 resistance and for any auxotrophic markers carried by the strain to ensure contamination had not occured.

8.3.4. Production of Colicin Ib.

Production of Colicin Ib was detected by exposing a Luria agar plate containing colonies of the test strain to chloroform vapour for twenty minutes to kill the bacteria. The chloroform vapour was allowed to evaporate away for a further 30 minutes before the plate was overlaid with 3 ml soft nutrient agar containing 0.1 ml of a 1:20 dilution of an overnight culture of a colicin sensitive indicator strain. After a 5 to 6 hr incubation at 37°C, a clear halo of killing around the test strain indicated the production of colicin Ib. This test was used to detect ColIb plasmids which did not carry an antibiotic resistance marker.

8.4. Assay of β-galactosidase.

The methods used were modified from that described previously by Casaregola *et al.* (1982), except that cultures were grown in SGC media (M9

salts containing 0.4 % [wt/vol] glucose and 0.2 % [wt/vol] Casamino Acids). Overnight cultures were diluted to OD_{600} of 0.05 and in the case of *recA730* (GY7221) and MC4100 strains, grown to OD_{600} of 0.35 at 37°C with aeration and assayed for β-galactosidase activity. The *recA441* strains (GC4597) were grown to OD_{600} of 0.35, at 30°C, then transferred to 42°C, adenine added to 0.5mM and the cultures incubated for a further 1 hr with aeration.

Prior to assaying β-galactosidase activity the OD_{600} of the cultures was measured using a Unicam spectophotometer. The assay was set up in 2.0 ml volumes. Tubes were set up with 0.2 ml culture and 1.8 ml Z-buffer (60 mM Na_2HPO_4; 40 mM NaH_2PO_4; 10 mM KCl; 1 mM $MgSO_4$). To these tubes was added 20 µl toluene which was mixed vigorously for 10 seconds to lyse the cells. The toluene was evaporated by incubating at 37°C for 2 hr. Once the toluene had evaporated, tubes were transferred to 28°C and 0.4 ml of 4 mg/ml ONPG (ortho-nitryl-phenyl-galactoside) added. When the ONPG indicator had changed to a pale yellow the reaction was terminated by the addition of 1.0 ml 1 M $NaCO_3$ and the time taken for the colour reaction recorded. The OD_{420} and OD_{550} readings were then taken using the Unicam spectophotometer. The specific activity of β-galactosidase was then calculated using the following formula:

$$\text{Specific Activity} = \frac{\dfrac{((A_{420} - (1.75 \times A_{550}))}{(0.0075 \times t)} \times \dfrac{r}{v}}{(A_{600} \times 0.227)}$$

Where t = time for reaction (seconds)

r = reaction volume (3.4 ml)

v = sample volume (0.2 ml)

8.5. DNA manipulations.

8.5.1. Small-scale plasmid DNA preparation.

The method used was a modification of the alkaline sodium dodecyl sulphate method of Birnboim and Doly (1979) and Ish-Howowicz and Burke (1981). 1.5 ml of an overnight culture of plasmid containing cells were centrifuged in a MSE microfuge for 1 min, and the supernatent removed. The pellet was resuspended in 100 µl of lysis buffer (25 mM Tris-HCl, pH 8.0; 10 mM EDTA, 50 mM sucrose). 200 µl of alkaline SDS (0.2 M NaOH; 1 % SDS) were then added, mixed gently and incubated on ice for 5 min. 150 µl of 3M potassium acetate solution (pH 4.8) were added, mixed vigorously and incubated on ice for a further 5 min.

The lysate was centrifuged for 3 min in a MSE microfuge and the supernatent transferred to a clean Eppendorf tube containing an equal volume of phenol/chloroform mix. The tube was mixed well and centrifuged for 5 min. The supernatent was transferred to a clean tube and the DNA precipitated by adding a tenth volume 2 M Sodium Acetate (pH 5.6) and two volumes of absolute ethanol. Following incubation at room temperature for 5 min, the DNA was pelleted for 3 min in a MSE microfuge. The pellet was washed with 70% ethanol, vacuum desiccated and resuspended in 40 µl distilled water.

Plasmid DNA for nucleotide sequencing was prepared as above with the addition of an ammonium acetate/ propan-2-ol precipitation step. The DNA was resuspended in a final volume of 100 µl. To this was added 33 µl of 10 M ammonium acetate, mixed vigorously and centrifuged for 10 min. The supernatent was transferred to a fresh tube containing an equal volume of propan-2-ol to precipitate the DNA, mixed and the DNA pelletted by centrifugation for a further 10 min in an MSE microfuge. The pellet was washed in 70 % ethanol, vaccum desiccated and resuspended in 20 µl

distilled water. This method allows miniprep plasmid DNA to be used conveniently used for nucleotide sequencing.

8.5.2. Restriction digest analysis.

DNA prepared by the alkaline SDS method was digested with restriction endonucleases in buffers supplied by, or recomended by the supplier (Bethesda Research laboratories, Inc.). Digests were routinely carried out in a volume of 25 µl, in the presence of 250 µg ml^{-1} RNaseA, and incubated at 37°C for 1 hr. For agarose gel electrophoresis, DNA samples were mixed with one fifth volume loading buffer (5 mM Tris-HCl, pH 7.5; 100 mg ml^{-1} glycerol; 0.01 mg ml^{-1} bromophenol blue). Restriction fragments were separated on HGT Seakem agarose horizontal slab gels of 0.7 - 1 % (w/v) in TAE buffer (40 mM Tris-acetate, pH 7.4; 1 mM EDTA) and run either overnight at 20V or for 3 hours at 100V in TAE buffer containing 5 µg ml^{-1} ethidium bromide. The fragments were visualised on a short wavelength UV transilluminator and photographed (Kodak X-Omat, 15 seconds exposure, f 4.5). The size of the restriction fragments was determined by comparison with λ *Hind*III (23.13, 9.4, 6.56, 4.36, 2.32, 2.03, 0.56 kb) or phiX174 x *Hae*III molecular weight markers (1.35, 1.08, 0.87, 0.6, 0.31, 0.28, 0.27, 0.23, 0.19, 0.11, 0.072 kb).

8.5.3. Cloning procedures.

Vector DNA and the plasmid DNA to be ligated were cut with the appropriate restriction enzymes. Specific fragments were isolated from 0.7 - 1.0 % agarose gels by running onto dialysis membrane inserted into the gel. The membrane was then removed while a 100 V potential was maintained across the gel. DNA was then washed from the membrane with distilled water and by spinning in a MSE microfuge. All DNA used for ligations was phenol extracted and ethanol precipitated as described in section 8.5.1.

The concentration of DNA was estimated by comparison with a known concentration of λ marker DNA, by running a small sample on an agarose gel. Vector and plasmid DNA were then mixed to give a fragment ratio of 1:3 vector:plasmid, such that the final concentration of DNA was approximately 0.1 μg. The ligation reactions were carried out in a total volume of 20 μl containing 2 μl of 10x ligation buffer (500 mM Tris-HCl, pH 7.8; 100 mM MgCl2; 200 mM dithiothreitol [DTT]; 10 mM ATP; 500 μg ml^{-1} bovine serum albumin [BSA: Bethesda Research Laboratories (BRL), nuclease free]; stored at -80°C) and 5 units T4 DNA ligase (BRL). The mixture was incubated at room temperature overnight, and subsequently used to transform a suitable host strain.

8.5.4. Creating blunt ends from 5′ and 3′ overhangs using T4 DNA Polymerase.

In the presence of 5′ overhangs T4 DNA Polymerase fills in the overhang to generate blunt ends whereas in the presence of 3′ overhangs the enzyme removes bases from the protruding ends.

Reactions were carried out at 37°C in a volume of 20 μl and consisted of: 17 μl DNA solution, 2 μl 10 x react I buffer (BRL), 1 μl dNTPs (2 mM stock) and 2.5 units T4 DNA Polymerase. Incubation was for 15 min and the reaction terminated by placing at 70°C for 5 min. The solution was phenol extracted, the DNA ethanol precipitated and resuspended in distilled water.

8.5.5. Dephosphorylation of DNA fragments.

Following restriction digestion of DNA with an enzyme which produces single-stranded 5′ protruding ends, 5′ phosphate residues were removed by treatment with calf intestinal alkaline phosphatase (CIAP), where required, to prevent religation of the ends. 10 units of this enzyme were added directly to the restriction buffer and incubated at 37°C for 30 min.

The enzymes were removed by extraction with phenol and the DNA ethanol precipitated before subsequent manipulations.

8.6. Strain manipulations.

8.6.1. Bacterial conjugation in liquid culture.

For routine of conjugative plasmids between bacterial strains and for tests of conjugative efficiency, bacteria from overnight culture were grown for approximately three mass doublings from OD_{600} of 0.05 to 0.35. Donor and recipient strains were mixed in a ratio of 1:1 (vol/vol) unless otherwise stated in a final volume of 3 ml in a sterile 50 ml flask. For routine strain constructions, the conjugation was allowed to proced for 1 hr for derepressed plasmids or 4 hr for wild type plasmids, before streaking out the culture on selective media. For quantitative mating experiments, 0.3 ml samples were taken at appropriate times, added to 2.7 ml sterile phosphate buffer and interupted in a mechanical agitator for 10 sec. 0.1 ml samples serially diluted were then plated out onto media to select for recipients containing the transferred plasmid.

All mating cultures were shaken gently (speed 1.5 in a New Brunswick gyratory waterbath) at 37°C unless otherwise stated. Parental cultures were also plated out onto selective media as a control.

8.6.2. Transformation.

Transformation was by a modification of the method of Cohen *et al.* (1972).

a) Preparation of competent cells.

Overnight culture of strains were diluted to OD_{600} of 0.025 and grown with aeration to OD_{600} of 0.35, with antibiotic selection where appropriate. Cells were pelleted in an MSE chillspin at 4000 rpm, 0°C for 8 min. The

supernatent was poured off and the cells washed twice by centrifugation as above in 5 ml of ice cold 0.1 M $CaCl_2$. The pellet was resuspended in 1 ml ice cold 0.1 M $CaCl_2$. These cells could be kept overnight at 0°C with no loss of transformation efficiency.

b) Uptake of DNA.

250 µl of competent cells were used for each transformation. Approximately 0.1 - 0.5 µg DNA was added to these cells in a strerile Eppendorf tube and the contents mixed gently. The cells were then kept on ice for 1 hr, heat shocked at 42°C for 3 min and then placed back on ice for 10 min. The contents of the tube were then transferred to 5 ml prewarmed Luria broth and incubated for 1 hour at an appropriate temperature (30°C or 37°C) in a shaking water bath. The cells were then pelleted in an MSE centrifuge and resuspended in 0.3 ml phosphate buffer. Dilutions were plated out on selective media to give single transformants.

8.6.3. Electroporation.

This method uses high voltage to force DNA into the cells. In order to subject the cells to a high voltage it is necessary that the cells be in a solution of low electrical resistance, i.e in a salt free environment.

Overnight culture was used to innoculate 100 ml of fresh Luria broth to OD_{600} of 0.05 and grown to OD_{600} of 0.8. The cells were harvested by centrifugation in an MSE chillspin at 4°C, 4000 rpm and washed in an equal volume of cold sterile distilled water. The cells were repelleted and washed initially in 1/2 and then 1/50th of the original volume with cold water. Finally the pelleted cells were resuspended in 1/500th of the original volume with cold water. 40 µl of the concentrated cells were mixed with 1 to 100 ng of plasmid DNA and subjected to 1500 volts (capacitance set at 25 µF) in an electroporation cuvette with a 2 mm gap between electrodes using the Biorad electroporator apparatus.

1 ml of SOC medium was added immediately and the cells placed at 37°C with shaking for 1 hr. Serial dilutions of the culture were plated out on selective media to give single colonies.

The DNA used for electroporation also has to be in a salt free environment, consequently ligation mixes were ethanol precipitated, with the addition of 1 μg of tRNA to ensure quantitative precipitation of DNA prior to electroporation.

8.6.5. Selective strain lysis procedure using phage T6.

This procedure was used to selectively lyse one of the parental strains following a conjugation experiment. Phage T6 (3 x 10^{10} particles) were added to a 3 ml mating mixture and incubated at 37°C for 5 min, and then DNase I and RNaseA (150 and 100 μg/ml, respectively) for five min, with proteinase K (60 μg/ml) for 20 min, and finally with Brij 58 (5 mg/ml) at 0°C for 5 min. T6 resistant cells were harvested and washed by three rounds of centrifugation in a Sorvall SS34 rotor at 7, 000 rpm for 5 min with resuspension in ice-cold phosphate buffer containing Brij 58 (0,5 %). Two optical density (OD_{600}) units of MC4100 $T6^r$ cells were added to aid pelleting. Harvested cells were resuspended in 2 ml of Z buffer and assayed for β-galactosidase activity.

8.7.1. Isolation of M13 transformants.

E. coli F′ strain NM522 was transformed with the required M13 double stranded clone by either the $CaCl_2$ or electroporation method. 100 to 200 μl of the transformed cells were then plated out immediately by adding to 3 ml of soft nutrient agar, seeded with 100 μl of an overnight culture of the *E. coli* F′ strain. The mixture was vortexed briefly and poured on top of a minimal media agar plate. After incubating overnight at 37°C, individual

plaques were transferred to 1 ml of Lambda buffer with a strerile toothpick and stored at 4°C as phage stocks.

8.7.2 Isolation of single-stranded DNA.

Single-stranded M13 derived DNA was obtained by using 100 µl of phage stocks to infect 1.5 ml of Luria broth, seeded with 15 µl of an overnight culture of NM522 F′. The culture was shaken vigorously for 6 hr at 37°C. The cells were pelleted (13 K, 10 minutes) and the phage precipitated from 1 ml of supernatent by the addition of 200 µl of 20 % PEG (Polyethylene Glycol 6000) / 2.5 M NaCl, mixing and leaving at 15°C for 15 min. The phage was pelleted by spinning at 13, 000 rpm for 20 min in an Eppendorf microfuge and the supernatent completely removed using a drawn out Pasteur pipette and suction. The pellet was resuspended in 100 µl distilled water and the protein coat removed by phenol extraction. The single-stranded DNA, after the addition of 10 µl of 3 M sodium acetate, was then ethanol precipitated and resuspended in 20 µl of distilled water.

8.8. Nucleotide sequence determination.

Sequencing was performed by the dideoxynucleotide chain termination method on either single-stranded M13 recombinant DNA (Sanger *et al.*, 1980) or denatured double-stranded plasmid DNA using the Sequenase version 2.0 kit (United States Biochemical Corporation) under the recommended conditions. Samples of the completed reactions were electrophoresed on acrylamide gels (6 % acrylamide, 0.32 % bisacrylamide and 42.4% urea) in TBE buffer. Gels were dried and used to expose X-ray film from which the DNA sequence could be read. Primers were: A: the M13 universal primer and 18-mers complementary to *psiB* sequences: B: 5′ ATCATCCATATCCTGATG (residues 550 to 567 in Fig. 2.11.), C: 5′ CATCAGGATATGGATGAT (residues 567 to 550) and D:

ACTGCCATACTCGCCATT (residues 315 to 298). Programs obtained from the University of Wisconsin Genetics Computer Group were used for data analysis (Devereux *et al.*, 1984).

8.9. Southern Hybridization.

8.9.1. Oligolabelling of DNA fragments.

The method used was based on that of Feinberg and Vogelstein (1983). DNA fragments generated by restriction digestion were separated by electrophoresis in a 0.6 % low gelling temperature agarose gel (Seakem). The desired fragment, visualised using a UV transilluminator, was excised from the gel in a minimum volume and placed in a preweighed Eppendorf tube. The volume of agarose was determined by weight (1 mg = 1 µl). Water was added to the gel in the ratio of 1.5 µl water to 1 µl gel, the tube placed in a boiling water bath for 7 min followed by a 37°C water bath for 10 min.

The labelling reaction, carried out at room temperature, required the following reagents to be added in the order given: distilled water to a total volume of 20 µl; 3 µl oligolabelling buffer (see below); 0.6 µl BSA (10 mg ml^{-1} enzyme grade, BRL); DNA (5 to 25 ng); 1 µl ^{32}P-dCTP and 0.6 ml Klenow polymerase (1 unit μl^{-1}, Pharmacia).

Oligolabelling buffer is a mixture of solutions A,B and C in a ratio of 2:5:3, stored at -20°C. Solution A was 1.2 M Tris-HCl, pH 8.0; 120 mM $MgCl_2$; 1.7 % (vol/vol) β–mercaptoethanol; 0.5 mM dATP, dGTP, dTTP (Sigma: each 0.1 M triphosphate stock dissolved in 3 mM Tris-HCl; 0.2 mM EDTA, pH 7.0). Solution B was 2 M HEPES, titrated to pH 6.6 with NaOH. Solution C was an even suspension of hexadeoxynucleotides (Pharmacia) in 3 mM Tris-HCl; 0.2 mM EDTA, pH 7.0; at 90 OD units ml^{-1}.

The labelling reaction could be conveniently run overnight, but the reaction reaches a plateau after 3 hr. The reaction was stopped by adding 75

µl of stop solution (20 mM NaCl; 20 mM Tris-HCl, pH 7.5; 2 mM EDTA; 0.25 % (w/v) SDS).

8.9.2. Acid/Alkali denaturation of gels and blotting procedure.

This method is essentially as described by Southern (1980), except that hybridization was onto a Hybond nylon membrane (Amersham International). Following blotting, the filter was air dried and the DNA fixed onto the membrane by baking at 80°C for 2 hr.

8.9.3. In situ hybridization screening of bacterial colonies.

Following transformation, single colonies were picked from fresh selection plates and were transferred to equivalent positions on a grid on each of a) a 'master' selection plate and b) a Hybond nylon filter laid onto a selection plate. The master plate and filter-bearing plate were incubated at an appropriate temperature to allow colonies to grow.

Colony lysis was carried out according to Grunstein and Hogness (1975). The filter was peeled from the plate and was placed on a sheet of Whatman 3MM chromatography paper soaked in 0.5 M NaOH for 10 min during which the colonies lyse. The filter was then transferred to a sheet soaked in 1 M Tris-HCl pH 8.0 for 10 min, then a sheet soaked in 1 M Tris-HCl pH 8.0, 1.5 M NaCl for 10 min. The filter was then air dried and baked at 80°C for 2 hours.

8.9.4. Hybridization.

The method used for the hybridization step was based on that of Reed and Mann (1985). The filter was initially washed in prehybridization mix for 1 hr at 65°C. Prehybridization mix consisted of 450 mM NaCl; 3 mM Na2HPO4, pH 7.4; 3 mM EDTA; 1 % (w/v) SDS; 0.5 % (w/v) MARVEL milk powder (Cadbury); and 6 % PEG 6000. Approximately 0.1 µg of denatured

oligolabelled probe was added at this stage and hybridization allowed to proceed overnight at 65°C with gentle agitation. Excess labelled DNA was removed by washing repeatedly in 3 x SSC, 0.1 % SDS at 65°C untill the final wash removed no detectable radioactivity. Stringency was determined by the concentration of SSC in the final washes. Routiely this was 0.5 x SSC, 0.1 % SDS at 65°C. Filters were air dried and covered in cling film prior to autoradiography using Kodak X-Omat X-ray film.

8.10. SDS-polyacrilamide gel electrophoresis.

8.10.1. Preparation of whole cell extracts.

Whole cell extracts were prepared from five OD units (450 nm) of growing cells. For cells harbouring pBS215-1, extracts were prepared following a 1 hr induction with 1 mM IPTG. Washed cells were suspended in 75 µl of sample buffer (62.5 mM Tris-HCl, pH 6.8; 3 % SDS, 10 % glycerol, 5 % β-mercaptoethanol), boiled for 5 minutes and centrifuged at 13,000 rpm in an Eppendorf microfuge.

8.10.2. SDS-PAGE.

SDS-polyacrylamide gel electrophoresis was performed using gels containing 10 % (w/v) acrylamide (monomer:dimer ratio, 30:0.8). The procedure used was that of Laemmli, 1970, using a Biorad Protean II system. 30 µl of sample were loaded per track and run at 30 mA untill the dye front reached the end of the gel. Molecular weight markers (Sigma Chemical Company Ltd, U.K) were rabbit muscle myosin (205 kD), β-galactosidase (116 kD), phosphorylase b (97 kD) and bovine serum albumin (66 kD). Polypeptides were visualised by Coomassie blue staining.

10 % Separating gel

H_2O	7.9 ml
30% acrylamide mix	6.7 ml
1.5 M Tris (pH 8.8)	5.0 ml
10 % SDS	0.2 ml
10 % ammonium persulphate	0.2 ml
TEMED	8 µl

5 % Stacking gel

H2O	2.7 ml
30% acrylamide mix	0.67 ml
1.0 M Tris (pH 6.8)	0.5 ml
10 % SDS	40 µl
10 % ammonium persulphate	40 µl
TEMED	4 µl

Tris-glycine electrophoresis buffer:

25 mM Tris, 250 mM glycine (electrophoresis grade, pH 8.3), 0.1 % SDS.

Appendix.

A major theme of this thesis is the extent of similarity between ColIb and the F-like plasmids. The two experiments described in this appendix concern this similarity. It is known that the conjugation systems specified by ColIb and the F-like plasmids are distinct (Rees *et al.*, 1987). However, there are significant homologies between the leading regions of these plasmid groups. It has been demonstrated that ColIb carries *ssb* and *psiB* genes which are ~84 % identical to the corresponding genes of F (Howland *et al.*, 1989; this work). The F-like plasmids are known to encode a plasmid maintenance system in their leading region. Initially described in R1, this locus is known as *parB* on R1, or *flm* (F leading maintenance) on F and specifies *hok* and *sok* gene products (Gerdes *et al.*, 1986; 1988; Loh *et al.*, 1988). Appendix I describes an experiment in which the R1 *hok* gene was used as a probe in a Southern hybridization to determine whether ColIb carries a similar locus.

Plasmids which carry *ssb* and *psiB* genes belong to a group of plasmids which also show homology to the RepFIC replicon of plasmid F (Golub *et al.*, 1986; Couturier *et al.*, 1988). Techniques of replicon tying have allowed the relationships between plasmids to be examined by DNA-DNA hybridization using probes from the basic replicons of plasmids, rather than by classical incompatibility testing. The RepFIC replicon on F-like plasmids is located adjacent to one end of the Tra region (Fig.1.1). The ColIb Rep region, including the origin of replication (*oriV*) and the incompatibility/replication functions (*inc/rep*) had been mapped genetically to the S5 fragment of ColIb (Figure 1.2; Rees *et al.*, 1987) Appendix II describes a replicon typing experiment, carried out to determine whether the ColIb Rep region located by genetic methods was also the region that shows homology to RepFIC.

I. ColIb does not encode a *hok/sok* locus.

A Southern hybridization experiment was performed using the R1 *hok* gene as a probe, carried on a 0.3 kb *EcoRI/BamHI* fragment from pPR341 (Gerdes *et al.*, 1986). The test plasmids used were F (x*EcoRI*) as a positive control, ColIb (x*SalI*), pLG2001 (x*PstI*), and pALS4 (x*PstI*). The final stringency wash used was 1 x SSC 0.1% SDS. The R1 *hok* probe hybridized to the 11.1 kb E3 *EcoRI* fragment of plasmid F, but there was no hybridization observed to the ColIb DNA even after a week long exposure to X-ray film (Fig.1). It was concluded that the extent of homology between the leading region of ColIb and the F-like plasmids does not extend to include *hok/sok* although it is possible that the locus has diverged to such an extent that it was not picked up by hybridization at the stringency used.

II. Replicon typing of ColIb

A Southern hybridization experiment was performed using a 0.96 kb *EcoRI/HindIII* fragment from the RepFIC replicon carried on a recombinant plasmid, pULB2440 (Couturier *et al.*, 1988). The test plasmids used were ColIb (x *EcoRI*), ColIb (x *SalI*), pCRS5 (x *PstI*), and F (x *EcoRI*).

Figure 2 shows that the RepFIC probe hybridised to the 20 kb E2 (*EcoRI*) and 5 kb S5 (*SalI*) fragments of ColIb and to a 2.5 kb *EcoRI* fragment of plasmid F. The probe also hybridised to the 5.55 kb and 2.35 kb *PstI* fragments of pCRS5 with stronger hybridization to the 5.55 kb band. pCRS5 carries the S5 *SalI* ColIb fragment inserted into pBR328 (Fig.3.). These results would suggest that the *inc/rep* region mapped using genetic techniques by Rees *et al.* (1987) is related to the RepFIC replicon of IncFI plasmids.

Fig.1. Results of a Southern hybridization using a 0.3 kb *Eco*RI/*Bam*HI fragment carrying the RI *hok* gene to restriction fragments of plasmids ColIb and F.

Lanes show hybridization to (1) F x *Eco*RI, (2) pLG2001 x *Pst*I, (3) pALS4 x *Pst*I and (4) ColIb x *Sal*I separated by agarose gel electrophoresis.

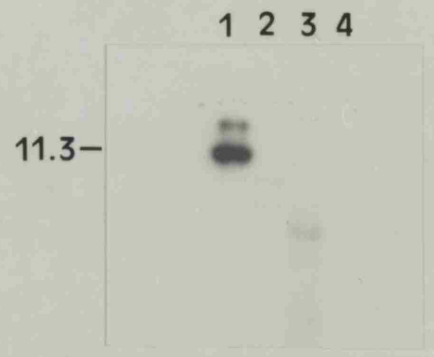


Fig.2. Results of a Southern hybridization using a 0.96 kb *Eco*RI/*Hind*III fragment carrying part of the RepFIC replicon from an FI plasmid.

Lanes show hybridization to (1) ColIb x *Eco*RI, (2) ColIb x *Sal*I, (3) pCRS5 x *Pst*I and (4) F x *Eco*RI separated by agarose gel electrophoresis.

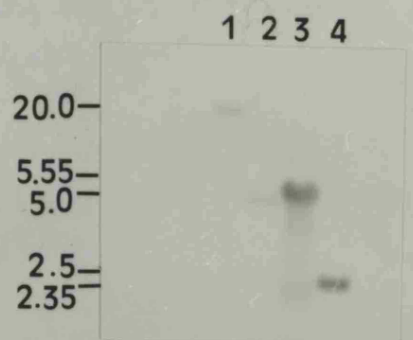
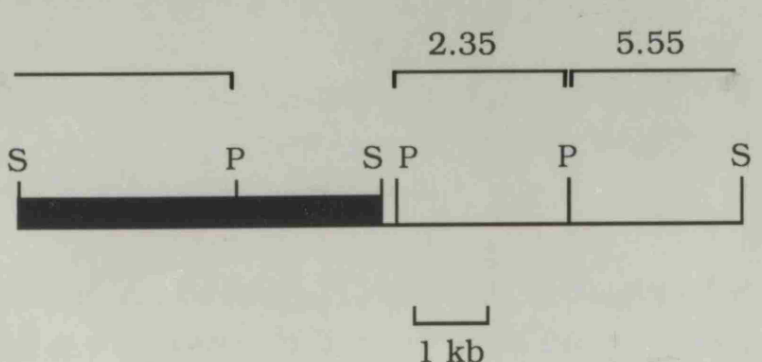


Fig.3. Restriction map of pCRS5.

Above the map, the 2.35 kb and 5.5 kb *Pst*I fragments that hybridized to the RepFIC probe are indicated. The plasmid is linearized at one end of the pBR328 vector (black box). Restriction sites are *Pst*I (P) and *Sal*I (S).



Literature Cited

Abdel-Monem, M., Taucher-Scholz, G., and Klinkert, M.Q. (1983). Identification of *Escherichia coli* DNA helicase I as the *traI* gene product of the F sex factor. Proc. Natl. Acad. Sci. U.S.A. **80**: 4659-4663.

Achtman, M., Willetts, N., and Clark, A.J. (1972). Conjugational complementation analysis of transfer-deficient mutants of F*lac* in *Escherichia coli*. J. Bacteriol. **110**: 831-842.

Alazard, R.J. (1983). Study of the expression of UVRA and SSB proteins *in vivo* in λ hybrid phages containing the *uvrA* and *ssbA* genes of *Escherichia coli*. Mutation. Res. **109**: 155-168.

Al-Doori, Z., Watson,M., and Scaife, J. (1982). The orientation of transfer of the plasmid RP4. Genet. Res. Camb. **39**: 99-103.

Backendorf, C., Brandsma, J.A., Kartasova, T., and van de Putte, P. (1983). *In vivo* regulation of the *uvrA* gene: role of the "-10" and "-30" promoter regions. Nucl. Acids Res. **11**: 5795-5810.

Bagdasarian, M., D'Ari, R., Filipowicz, W., and George, J. (1980). Suppression of induction of SOS functions in an *Escherichia coli tif-1* mutant by plasmid R100.1. J. Bacteriol. **141**: 464-469.

Bagdasarian, M., Bailone, A., Bagdasarian, M.M., Manning, P.A., Lurz, R., Timmis, K.N., and Devoret, R. (1986). An inhibitor of SOS induction, specified by a plasmid locus in *Escherichia coli*. Proc. Natl. Acad. Sci. USA. **83**: 5723-5726.

Bagg, A., Kenyon, C.J., and Walker, G.C. (1981). Inducibility of a gene product required for UV and chemical mutagenesis in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **78**: 5749-5753.

Bailone, A., Backman, A., Sommer, S., Celerier, J., Bagdasarian, M.M., Bagdasarian, M., and Devoret, R. (1988). PsiB polypeptide prevents activation of RecA protein in *Escherichia coli*. Mol. Gen. Genet. **214**: 389-395.

Barlett, M.M., Erickson, M.J., and Meyer, R.J. (1990). Recombination between directly repeated origins of conjugative transfer cloned in M13 bacteriophage DNA models ligation of the transferred plasmid strand. Nucl. Acids Res. **18**: 3579-3586.

Barany, F. (1985). Single-stranded hexameric linkers: a system for in-phase insertion mutagenesis and protein engineering. Gene **37**: 111-123.

Birnboim, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**: 1513-1523.

Blanco, M., Herrera, G., Collado, P., Rebollo, J., and Botella, L.M. (1982). Influence of RecA protein on induced mutagenesis . Biochimie **64**: 633-636.

Boulnois, G.J. (1981). Colicin Ib does not cause plasmid-promoted abortive phage infection of *Escherichia coli* K-12. Mol. Gen. Genet. **182**: 508-510.

Boulnois, G.J., and Wilkins, B.M. (1978). A ColI-specified product, synthesized in newly infected recipients, limits the amount of DNA transferred during conjugation of *Escherichia coli* K-12. J. Bacteriol. **133**: 1-9.

Boulnois, G.J., Wilkins, B.M., and Lanka, E. (1982). Overlapping genes at the primase locus of the large plasmid ColI. Nucl. Acids. Res. **10**: 855-869.

Bradley, D.E. (1983). Derepressed plasmids of incompatibility group I_1 determine two different morphological forms of pilus. Plasmid **9**: 331-334.

Bradley, D.E. (1984). Characteristics and function of thick and thin conjugative pili determined by transfer-derepressed plasmids of incompatibility groups I_1, I_2, I_5, B, K and Z. J. Gen. Microbiol. **130**: 1489-1502.

Bradshaw, H.D. Jr., Traxler, B.A., Minkley, E.G. Jr., Nester, E.W., and Gordon, M.P. (1990). Nucleotide sequence of the *traI* (helicase I) gene from the sex factor F. J. Bacteriol. **172**: 4127-4131.

Brandsma, J.A., Bosch, D., Backendorf, C., and van de Putte, P. (1983). A common regulatory region shared by divergently transcribed genes of the *Escherichia coli* SOS system. Nature **305**: 243-245.

Brandsma, J.A., Bosch, D., de Ruyter, M., and van de Putte, P. (1985). Analysis of the regulatory region of the *ssb* gene of *Escherichia coli*. Nucl. Acid. Res. **13**: 5095-5109.

Brasch, M.A., and Meyer, R.J. (1987). A 38 base-pair segment of DNA is required in *cis* for conjugative mobilization of broad-host-range plasmid R1162. J. Mol. Biol. **198**: 361-369.

Brent, R., and Ptashne, M. (1981). Mechanism of action of the *lexA* gene product. Proc. Natl. Acad. Sci. U.S.A. **78**: 4204-4208.

Bridges, B.A., and Woodgate, R. (1985). Mutagenic repair in *Escherichia coli*: Products of the *recA* gene and of the *umuD* and *umuC* genes act at different steps in UV-induced mutagenesis. Proc. Natl. Acad. Sci. U.S.A. **82**: 4193-4197.

Burckhardt, S.E., Woodgate, R., Scheuermann, R.H., and Echols, H. (1988). UmuD mutagenesis protein of *Escherichia coli*: Overproduction, purification, and cleavage by RecA. Proc. Natl. Acad. Sci. USA. **85**: 1811-1815.

Casadaban, M.J. (1976). Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104**: 541-555.

Casaregola, S., D'Ari, R., and Huisman, O. (1982). Quantitative evaluation of *recA* gene expression in *Escherichia coli*. Mol. Gen. Genet. **185**: 430-439.

Castellazzi, M., George, J., and Buttin, G. (1972). Prophage induction and cell division in *E. coli*. I. Further characterization of the thermosensitive mutation *tif-1* whose expression mimics the effect of UV-irradiation. Mol. Gen. Genet. **119**: 139-152.

Cavalli-Sforza, L.L., Lederberg, J., and Lederberg, E. (1953). An infective factor controlling sex compatibility on *Bacterium coli*. J. Gen. Microbiol. **8**: 89-103.

Chang, A.C.Y., and Cohen, S.N. (1978). Construction and characterisation of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**: 1141-1156.

Chase, J.W., Merrill, B.M., and Williams, K.R. (1983). F sex factor encodes a single-stranded DNA binding protein (SSB) with extensive sequence homology to *Escherichia coli* SSB. Proc. Natl. Acad. Sci. USA. **80**: 5480-5484.

Chase, J.W., Murphy, J.B., Whittier, R.F., Lorensen, E., and Sninsky, J.J. (1983). Amplification of ssb-1 mutant single-stranded DNA-binding protein in *Escherichia coli*. J. Mol. Biol. **164**: 193-211.

Chase, J.W., and Williams, K.R. (1986). Single-stranded DNA binding proteins required for DNA replication. Ann. Rev. Biochem. **55**: 103-136.

Chatfield, L.K., Orr, E., Boulnois, G.J., and Wilkins, B.M. (1982). DNA primase of ColIb is involved in conjugal DNA synthesis in donor and recipient bacteria. J. Bacteriol. **152**: 1188-1195.

Chatfield, L.K., and Wilkins, B.M. (1984). Conjugative transfer of IncI1 plasmid DNA primase. Mol. Gen. Genet. **197**: 461-466.

Chaudhury, A.M., and Smith, G.R. (1985). Role of *E. coli* RecBC enzyme in SOS induction. Mol. Gen. Genet. **201**: 525.

Cheah, K-C., and Skurray, R. (1986). The F plasmid carries an IS3 insertion within *finO*. J. Gen. Microbiol. **132**: 3269-3275.

Clark, A. J. (1967). The beginning of a genetic analysis of recombination-proficiency. J. Cell. Physiol. **70**: 165-180.

Cohen, S.N., Chang, A.C.Y., and Hsu, L. (1972). Nonchromosomal antibiotic resistance in bacterial genetic transformation of *Escherichia coli* R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69**: 2110-2114.

Cohen, S.P., Resnick, J., and Sussman, R. (1983). Interaction of single-strand binding protein and recA protein at the single-stranded DNA site. J. Mol. Biol. **167**: 901-909.

Couturier, M., Bex, F., Bergquist, P.L., and Maas, W.K. (1988). Identification and classification of bacterial plasmids. Microbiol. Rev. **52**: 375-395.

Craig, N.L., and Roberts, J.W. (1980). *E.coli* recA protein-directed cleavage of phage λ repressor requires polynucleotide. Nature. **283**: 26-30.

Cram, D., Ray, A., O'Gorman, L., and Skurray, R. (1984). Transcriptional analysis of the leading region in F plasmid DNA transfer. Plasmid **11**: 221-233.

D'Ari, R., and Huisman, O. (1982). DNA replication and indirect induction of the SOS response in *Escherichia coli*. Biochimie. **64**: 623-627.

D'Ari, R., and Huisman, O. (1983). Novel mechanism of cell division inhibition associated with the SOS response in *Escherichia coli*. J. Bacteriol. **156**: 243-250.

Datta, N., Lawn, A.M., and Meynell, E. (1966). The relationship of F type piliation and F phage sensitivity to drug resistance transfer in R^+F^- *E. coli* K12. J. Gen. Microbiol. **45**: 365-376.

Devereux, J.P., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids. Res. **12**: 387-395.

Di Laurenzio, L., Frost, L.S., Finlay, B.B., and Paranchych, W. (1991). Characterization of the *oriT* region of the IncFV plasmid pED208. Mol Microbiol. **5**:

Dorman, C.J. (1991). DNA supercoiling and environmental regulation of gene expression in pathogenic bacteria. Infection and Immunity. **59**: 745-749.

Dowden, S.B., and Strike, P. (1982). R46-derived recombinant plasmids affecting DNA repair and mutation in *E.coli*. Mol. Gen. Genet. **186**: 140-144.

Dowden, S.B., Cossins, L., Glazebrook, J.A., Ramsden, M., and Strike, P. (1982). DNA-damage inducible genes on the I group plasmid TP110. Biochimie. **64:** 681-685.

Dowden, S.B., Glazebrook, J.A., and Strike, P. (1984). UV inducible UV protection and mutation functions on I group plasmid TP110. Mol. Gen. Genet. **193:** 316-321.

Drlica, K., and Snyder, M. (1978). Superhelical *Escherichia coli* DNA: relaxation by coumermycin. J. Mol. Biol. **120:** 145-154.

Duckworth, D.H., and Pinkerton, T.C. (1988). ColIb plasmid genes that inhibit the replication of T5 and T7 bacteriophage. Plasmid **20:** 182-193.

Dutreix, M., Backman, A., Celerier, J., Bagdasarian, M.M., Sommer, S., Bailone, A., Devoret, R., and Bagdasarian, M. (1988). Identification of *psiB* gene of plasmids F and R6-5. Molecular basis for the *psiB* enhanced expression in plasmid R6-5. Nucl. Acids. Res. **16:** 10669-10679.

Elledge, S.J., and Walker, G.C. (1983a). Proteins required for ultraviolet light and chemical mutagenesis: identification of the products of the *umuC* locus of *Escherichia coli*. J. Mol. Biol. **164:** 175-192.

Elledge, S.J., and Walker, G.C. (1983b). The *muc* genes of pKM101 are induced by DNA damage. J. Bacteriol. **155:** 1306-1315.

Ennis, D.G., Fisher, B., Edmiston, S., and Mount, D.W. (1985). Dual role for *Escherichia coli* RecA protein in SOS mutagenesis. Proc. Natl. Acad. Sci. **82:** 3325-3329.

Everett, R., and Willetts, N. (1980). Characterization of an *in vivo* system for nicking at the origin of conjugal DNA transfer of the sex factor F. J. Mol. Biol. **136:** 129-150.

Falklow, S., Hedges, R.W., Guerry, R., and Datta, N. (1974). Polynucleotide sequence relationships among plasmids of the I compatibility complex. J. Gen. Microbiol. **85:** 65-76.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabelling DNA restriction fragments to a high specific activity. Anal. Biochem. **132**: 6-13.

Finnegan, D.J and Willetts, N.S. (1971). Two classes of F*lac* mutants insensitive to transfer inhibition by an F-like R factor. Mol. Gen. Genet. **111**: 256-264.

Finnegan, D., and Willetts, N. (1973). The site of action of the F transfer inhibitor. Mol. Gen. Genet. **127**: 307-316.

Flory, J., and Radding, C.M. (1982). Visualisation of RecA protein and it's association with DNA: A priming effect of single-strand-binding protein. Cell **28**: 747-756.

Fu, Y. -H.F., Tsai, M-M., Luo, Y., and Deonier, R.C. (1991). Deletion analysis of the F plasmid *oriT* locus. J. Bacteriol. **173**: 1012-1020.

Furuya, N., Nisioka, T., and Komano, T. (1991). Nucleotide sequence and functions of the *oriT* operon in IncI1 plasmid R64. J. Bacteriol. **173**: 2231-2237.

Gellert, M., Mizuuchi, K., O'Dea, M.H., Itoh, T., and Tomizawa, J. (1977). Nalidixic acid resistance: A second genetic character involved in DNA gyrase activity. Proc. Natl. Acad. Sci. U.S.A. **74**: 4772-4776.

George, J., Devoret, R., and Radman, M. (1974). Indirect ultraviolet-reactivation of phage lambda. Proc. Natl. Acad. Sci. U.S.A. **71**: 144-147.

Gerdes, K., Rasmussen, P.B., and Molin, S. (1986). Unique type of plasmid maintenance function: Postsegregational killing of plasmid-free cells. Proc. Natl. Acad. Sci. USA. **83**: 3116-3120.

Gerdes, K., Helin, K., Christensen, O.W., and Lobner-Olesen, A. (1988). Translational control and differential RNA decay are key elements regulating postsegregational expression of the killer protein encoded by the *parB* locus of plasmid R1. J. Mol. Biol. **203**: 119-129.

Gerdes, K., Thisted, T., and Martinussen, J. (1990). Mechanism of post-segregational killing by the *hok/sok* system of plasmid R1: *sok* antisense RNA regulates formation of a hok mRNA species correlated with killing of plasmid free cells. Mol. microbiol. **4**: 1807-1818.

Gilbert, W., and Dressler, D. (1968). DNA replication: the rolling circle model. Cold Spring Harbor Symp. Quant. Biol. **33**: 473-484.

Glassberg, J., Meyer, R.R., and Kornberg, A. (1979). Mutant single-stranded binding protein of *Escherichia coli*: genetic and physiological characterization. J. Bacteriol. **140**: 14-19.

Glazebrook, J.A., Grewal, K.K., and Strike, P. (1986). Molecular analysis of the UV protection and mutation genes carried by the I incompatibility group plasmid TP110. J. Bacteriol. **168**: 251-256.

Glenn, J., and Duckworth, D. H. (1980). Fluorescence changes of a membrane-bound dye during bacteriophage T5 infection of *Escherichia coli*. J. Virol. **33**: 553-556.

Golub, E.I., and Brooks Low, K. (1985). Conjugative plasmids of enteric bacteria from many different incompatibility groups have similar genes for single-stranded DNA-binding proteins. J. Bacteriol. **162**: 235-241.

Golub, E.I., and Brooks Low, K. (1986a). Derepression of single-stranded DNA-binding protein genes on plasmids derepressed for conjugation, and complementation of an *E.coli ssb*$^-$ mutation by these genes. Mol. Gen. Genet. **204**: 410-416.

Golub, E.I., and Brooks Low, K. (1986b). Unrelated conjugative plasmids have sequences which are homologous to the leading region of the F factor. J. Bacteriol. **166**: 670-672.

Golub, E.I., Bailone, A., and Devoret, R. (1988). A gene encoding an SOS inhibitor is present in different conjugative plasmids. J. Bacteriol. **170**: 4392-4394.

Gottlieb, J.H., and Duckworth, D.H. (1983). Location of the *abi, col,* and *imm* genes of pHU011, a colicin Ib plasmid derivative. Gene **26**: 301-302.

Greenberg, J., Berends, L.T., Donch, J., and Green, H.M.L. (1974). *exrB*: a *malB*-linked gene in *Escherichia coli* B involved in sensitivity to radiation and filamentation. Genet. Res. **23**: 175-184.

Grindley, N.D.F., Humphreys, G.O., and Anderson, E.S. (1973). Molecular studies of R factor compatibility groups. J. Bacteriol. **115**: 387-398.

Grinter, N. (1981). Analysis of chromosome mobilization using hybrids between plasmid RP4 and a fragment of bacteriophage λ carrying IS*1*. Plasmid **5**: 267-276.

Gudas, L.J. (1976). The induction of protein X in DNA repair and cell division mutants of *Escherichia coli*. J. Mol. Biol. **104**: 567-587.

Gudas, L.J., and Pardee, A.B. (1975). Model for regulation of *Escherichia coli* DNA repair functions. Proc. Natl. Acad. Sci. U.S.A. **74**: 2330-2334.

Gupta, S.K., and McCorquodale, D.J. (1988). Nucleotide sequence of a DNA fragment that contains the *Abi* gene of the ColIb plasmid. Plasmid **20**: 194-206.

Harrington, L.C., and Rogerson, A.C. (1990). The F pilus of *Escherichia coli* appears to support stable DNA transfer in the absence of wall-to-wall contact between cells. J. Bacteriol. **172**: 7263-7264.

Hayes, W. (1953). Observations on a transmissible agent determining sexual differentiation in *Bacterium coli*. J. Gen. Microbiol. **8**: 72-88.

Horri, T., Ogawa, T., Nakatani, T, Hase, T., Matsubara, H., and Ogawa, H. (1981). Regulation of SOS functions: purification of *E. coli* LexA protein and determination of its specific site cleaved by the RecA protein. Cell **27**: 515-522.

Howard-Flanders, P., and Theriot, L. (1966). Mutants of *Escherichia coli* K-12 defective in DNA repair and in genetic recombination. Genetics **53**: 1137-1150.

Howarth, S. (1965). Resistance to the bactericidal effect of ultraviolet radiation conferred on enterobacteria by the colicin factor ColI. J. Gen. Microbiol. **40**: 43-55.

Howarth, S. (1966). Increase in frequency of ultraviolet induced mutation brought about by the colicin factor ColI in *Salmonella typhimurium*. Mutat. Res. **3**: 129-134.

Howland, C.J. (1989). The single-stranded DNA-binding protein gene of plasmid ColIb-P9. Ph.D. thesis, University of Leicester, U.K.

Howland, C.J., and Wilkins, B.M. (1988). Direction of conjugative transfer of IncI1 plasmid ColIb-P9. J. Bacteriol. **170**:

Howland, C.J., Rees, C.E.D., Barth, P.T., and Wilkins, B.M. (1989). The *ssb* gene of plasmid ColIb-P9. J. Bacteriol. **171**: 2466-2473.

Huisman, O., and D'Ari, R. (1981). An inducible DNA replication-cell division coupling mechanism in *E.coli*. Nature. **290**: 797-799.

Huisman, O., and D'Ari, R. (1983). Effect of suppressors of SOS-mediated filamentation on *sfiA* operon expression in *Escherichia coli*. J. Bacteriol. 153: 169-175.

Hull, R., and Moody, E.E. (1976). Isolation and genetic characterization of *Escherichia coli* K-12 mutations affecting bacteriophage T5 restriction by the ColIb plasmid. J. Bacteriol. **177**: 229-236.

Ihler, G., and Rupp, W.D. (1969). Strand-specific transfer of donor DNA during conjugation in *E. coli*. Proc. Natl. Acad. Sci. U.S.A. **63**: 138-143.

Ippen-Ihler, K.A., and Minkley, E.G. Jr. (1986). The conjugation system of F, the fertility factor of *Escherichia coli*. Annu. Rev. Genet. **20**: 593-624.

Ish-Horowicz, D., and Burk, J.F. (1981). Rapid and efficient cosmid cloning. Nucl. Acids Res. **9**: 2989-2998.

Kato, T., and Shinoura, Y. (1977). Isolation and characterization of mutants of *Escherichia coli* deficient in induction of mutations by ultraviolet light. Mol. Gen. Genet. **156**: 121-131.

Kenyon, C.J., and Walker, G.C. (1980). DNA-damaging agents stimulate gene expression at specific loci in *Escherichia coli*. Proc. Natl. Acad. Sci. USA. **77**: 2819-2823.

Kenyon, C.J., and Walker, G.C. (1981). Expression of the *E.coli uvrA* gene is inducible. Nature. **289**: 808-810.

Kim, K., and Meyer, R.J. (1989). Unidirectional transfer of broad-host-range plasmid R1162 during conjugative mobilization. Evidence for genetically distinct events at *oriT*. J. Mol. Biol. **208**: 501-505.

Kingsman, A., and Willetts, N. (1978). The requirements for conjugal DNA synthesis in the donor strain during F *lac* transfer. J. Mol. Biol. **122**: 287-300.

Kokotek, W., and Lotz, W. (1989). Construction of a *lacZ*-kanamycin-resistance cassette, useful for site-directed mutagenesis and as a promoter probe. Gene **84**: 467-471.

Kolodkin, A.L., Capage, M.A., Golub, E.I., and Brooks Low, K. (1983). F sex factor of *Escherichia coli* K-12 codes for a single-stranded DNA binding protein. Proc. Natl. Acad. Sci. USA. **80**: 4422-4426.

Komano, T., Funayama, N., Kim, S-R., and Nisioka, T. (1990). Transfer region of IncI1 plasmid R64 and role of shufflon in R64 transfer. J. Bacteriol. **172**: 2230-2235.

Kowalczykowski, S.C., and Krupp, R.A. (1987) Effects of *Escherichia coli* SSB protein on the single-stranded DNA-dependent ATPase activity of *Escherichia coli* RecA protein. Evidence that SSB protein facilitates the binding of RecA protein to regions of secondary structure within the single-stranded DNA. J. Mol. Biol. **193**: 97-113.

Krueger, J.H., and Walker, G.C. (1984). *groEL* and *dnaK* genes of *Escherichia coli* are induced by UV irradiation and nalidixic acid in an $htpR^+$ - dependent fashion. Proc. Natl. Acad. Sci. U.S.A. **81**: 1499-1503.

Lahue, E.E., and Matson, S.W. (1988). *Escherichia coli* DNA helicase I catalyses a unidirectional and highly processive unwinding reaction. J. Biol. Chem. **263**: 3208-3215.

Lahue, E.E., and Matson, S.W. (1990). Purified *Escherichia coli* F-factor TraY protein binds *oriT*. J. Bacteriol. **172**: 1385-1391.

Lahue, R.S., Au, K.G., and Modrich, P. (1989). DNA mismatch correction in a defined system. Science **245**: 160-164.

Lackey, D., Walker, G.C., Keng, T., and Linn, S. (1977). Characterisation of an endonuclease associated with the drug resistance plasmid pKM101. J. Bacteriol. **131**: 583-588.

Laemmli, U.K. (1987). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685.

Lanka, E., and Barth, P.T. (1981). Plasmid RP4 specifies a deoxyribonucleic acid primase involved in its conjugal transfer and maintenance. J. Bacteriol. **148**: 769-781.

Lanka, E., Scherzinger, E., Gunther, E., and Furste, J.P. (1979). A DNA primase specified by I-like plasmids. Proc. Natl. Acad. Sci. USA **76**: 3632-3636.

Lieberman, H.B., and Witkin, E.M. (1981). Variable expression of the *ssb-1* allele in different strains of *Escherichia coli* K-12 and B: differential suppression of its effect on DNA replication, DNA repair, and ultraviolet mutagenesis. Mol. Gen. Genet. **183**: 348-355.

Lieberman, H.B., and Witkin, E.M. (1983). DNA degradation, UV sensitivity and SOS-mediated mutagenesis in strains of *Escherichia coli* deficient in single-stranded DNA binding protein: Effects of mutations and treatments

that alter levels of Exonuclease V or RecA protein. Mol. Gen. Genet. **190**: 92-100.

Little, J.W. (1984). Autodigestion of lexA and phage λ repressors. Proc. Natl. Acad. Sci. U.S.A. **77**: 3225-3229.

Little, J.W., Edmiston, S.H., Pacelli, L.Z., and Mount, D.W. (1980). Cleavage of the *Escherichia coli lexA* protein by the *recA* protease. Proc. Natl. Acad. Sci. U.S.A. **77**: 3225-3229.

Lodwick, D., Owen, D., and Strike, P. (1990). DNA sequence analysis of the *imp* UV protection and mutation operon of the plasmid TP110: identification of a third gene. Nucl. Acids Res. **18**: 5045-5050.

Loh, S.M., Cram, D.S., and Skurray, R.A. (1988). Nucleotide sequence and transcriptional analysis of a third function (Flm) involved in F-plasmid maintenance. Gene **66**: 259-268.

Loh, S., Cram, D., and Skurray, R. (1989). Nucleotide sequence of the leading region adjacent to the origin of transfer on plasmid F and its conservation among conjugative plasmids. Mol. Gen. Genet 219: 177-186.

Loh, S., Skurray, R. Celerier, J., Bagdasarian, M., Bailone, A., and Devoret, R. (1990). Nucleotide sequence of the *psiA* (plasmid SOS inhibition) gene located on the leading region of plasmids F and R6-5. Nucl. Acids. Res.

Lu, A.-L., Welsh, K., Clark, S., Su, S.-S., and Modrich, P. (1984). Repair of DNA base-pair mismatches in extracts of *Escherichia coli*. Cold Spring Harbor Symp. Quant. Biol. **49**: 589-596.

Lu, C., Scheurmann, R.H., and Echols, H. (1986). Capacity of RecA protein to bind preferentially to UV lesions and inhibit the editing subunit (ε) of DNA polymerase III: A possible mechanism for SOS-induced targeted mutagenesis. Proc. Natl. Acad. Sci. U.S.A **83**: 619-623.

Lu, C., and Echols, H. (1987). RecA protein and SOS. Correlation of mutagenesis phenotype with binding of mutant RecA proteins to duplex DNA and LexA cleavage. J. Mol. Biol. **196**: 497-504.

Lundquist, P.D., and Levin, B.R. (1986). Transitory derepression and the maintenance of conjugative plasmids. Genetics **113**: 483-497.

Marsh, L., and Walker, G.C. (1987). New phenotypes associated with *mucAB*: Alteration of a MucA sequence homologous to the LexA cleavage site. J. Bacteriol. **169**: 1818-1823.

Matson, S.W., and Morton, B. S. (1991). *Escherichia coli* DNA helicase I catalyses a site and strand-specific nicking reaction at the F plasmid *oriT*. J. Biol. Chem. **266**: 109-114.

Maturin, L.J. Sr., and Curtiss, R III. (1981). Role of ribonucleic acid synthesis in conjugational transfer of chromosomal and plasmid deoxyribonucleic acids. J. Bacteriol. **146**: 552-563.

McCann, J., Spingarn, N.E., Kobori, J., and Ames, B.N. (1975). Detection of carcinogens as mutagens: bacterial tester strains with R factors plasmids. Proc. Natl. Acad. Sci. U.S.A. **72**: 979-983.

McEntee, K. (1977). Protein X is the product of the *recA* gene of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **74**: 5275-5279.

McEntee, K., and Weinstock, G.M. (1981). *tif-1* mutation alters polynucleotide recognition by the *recA* protein of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **78**: 6061-6065.

McEntee, K., Weinstock, G.M., and Lehman, I.R. (1980). RecA protein-catalysed strand assimilation: stimulation by *Escherichia coli* single-stranded DNA-binding protein. Proc. Natl. Acad. Sci. U.S.A. **77**: 857-861.

Menzel, R and Gellert, M. (1987). Fusions of the *Escherichia coli gyrA* and *gyrB* control regions to the galactokinase gene are inducible by coumermycin treatment. J. Bacteriol. **169**: 1272-1278.

Merryweather, A., Rees, C.E.D., Smith, N.M., and Wilkins, B.M. (1986). Role of *sog* polypeptides specified by plasmid ColIb-P9 and their transfer between conjugating bacteria. EMBO. J. **5**: 3007-3012.

Meyer, R., (1989). Site-specific recombination at *oriT* of plasmid R1162 in the absence of conjugative transfer. J. Bacteriol. **171**: 799-806.

Meyer, R.R., Glassberg, J., and Kornberg, A. (1979). An *Escherichia coli* mutant defective in single-strand binding protein is defective in DNA replication. Proc. Natl. Acad. Sci. U.S.A. **76**: 1702-1705.

Meyer, R.R., Voegele, D.W., Ruben, S.M., Rein, D.C., and Trela, J.M. (1982). Influence of single-stranded DNA-binding protein on *recA* induction in *Escherichia coli.* Mutat. Res. **94**: 299-313.

Meyer, R.R., and Laine, P.S. (1990). The single-stranded DNA-binding protein of *Escherichia coli.* Microbiol. Rev. **54**: 342-380.

Meynell, E., and Datta, N. (1965). Functional homology of the sex factor and resistance transfer factors. Nature **207**: 884-885.

Meynell, E., and Datta, N. (1966). The nature and incidence of conjugation factors in *E. coli.* Genet. Res. **7**: 141-148.

Meynell, E., and Datta, N. (1967). Mutant drug resistant factors of high transmissibility. Nature **259**: 455-458.

Meynell, E., Meynell, G.G., and Datta, N. (1968). Phylogenetic relationships of drug-resistance factors and other transmissible bacterial plasmids. Bacteriol. Rev. **32**: 55-83.

Miller, J.H. (1984). In *Experiments in molecular genetics.* Cold Spring Harbor, New York, Cold Spring Harbor Laboratory.

Monk, M. (1969). Induction of phage-lambda by transferred irradiated ColI DNA. Mol. Gen. Genet. **106**: 14-24.

Moreau, P.L., Pelico, J.V., and Devoret, R. (1982). Cleavage of λ repressor and synthesis of RecA protein induced by transferred UV-damaged F sex factor. Mol. Gen. Genet. **186**: 170-179.

Moreau, P.L., and Roberts, J.W. (1984). RecA protein-promoted λ repressor cleavage: complementation between RecA441 and RecA430 proteins in vitro. Mol. Gen. Genet. **198**: 25-34.

Moreau, P.L. (1987). Effects of overproduction of single-stranded DNA-binding protein on RecA protein-dependent processes in *Escherichia coli*. J. Mol. Biol. **194**: 621-634.

Moreau, P.L. (1988). Overproduction of single-stranded DNA-binding protein specifically inhibits recombination of UV-irradiated bacteriophage DNA in *Escherichia coli*. J. Bacteriol. **170**: 2493-2500.

Mount, D.W., Low, K.B., and Edmiston, S.J. (1972). Dominant mutations (*lex*) in *Escherichia coli* K-12 which affect radiation sensitivity and frequency of ultraviolet light-induced mutations. J. Bacteriol **112**: 886-893.

Mullineaux, P., and Willetts, N. (1985). Promoters in the transfer region of plasmid F. In Plasmids in Bacteria (D.R. Helinski, S.N. Cohen, D.B. Clewell, D.A. Jackson, and A. Hollaender, eds) pp. 605-614. Plenum, New York.

Neidhardt, F.C., Van Bogelen, R.A., and Lau, E.T. (1983). Molecular cloning and expression of a gene that controls the high-temperature regulon of *Escherichia coli*. J. Bacteriol. **153**: 597-603.

Nohmi, T., Battista, J.R., Dodson, L.A., and Walker, G.C. (1988). RecA-mediated cleavage activates UmuD for mutagenesis: Mechanistic relationship between transcriptional derepression and post-translational activation. Proc. Natl. Acad. Sci. USA. **85**: 1816-1820.

Ohki, M., and Tomizawa, J. (1968). Asymmetric transfer of DNA strands in bacterial conjugation. Cold Spring Harbor Symp. Quant. Biol. **33**: 651-657.

Osterlund, M., Luthman, H., Nilsson, S.V., and Magnusson, G. (1982). Ethidium Bromide inhibits restriction endonucleases cleave one strand of circular DNA. Gene **20**: 121-125.

Ou, J.T., and Anderson, T.F. (1970). Role of pili in conjugation. J. Bacteriol. **102**: 648-654.

Paoletti, C., Salles, B., and Giacomoni, P. (1982). An immunoradiometric quantitative assay of *Escherichia coli recA* protein. Biochemie. **64**: 239-246.

Pansegrau, W., and Lanka, E. (1991). Common sequence motifs in DNA relaxases and nick regions from a variety of DNA transfer systems. Nucl. Acids Res. **19**:

Perrino, F.W., Rein, D.C., Bobst, A.M., and Meyer, R.R. (1987). The relative rate of synthesis and levels of single-stranded DNA binding protein during induction of SOS repair in *Escherichia coli*. Mol. Gen. Genet. **209**: 612-614.

Perry, K.L., and Walker, G.C. (1982). Identification of plasmid (pKM101)-coded proteins involved in mutagenesis and UV resistance. Nature. **300**: 278-281.

Perry, K.L., Elledge, S.J., Mitchell, B.B., and Marsh, L. (1985). *umuDC* and *mucAB* operons whose products are required for UV light- and chemical-induced mutagenesis: UmuD, MucA, and LexA proteins share homology. Proc. Natl. Acad. Sci. USA. **85**: 4331-4335.

Peterson, K.R., Ossanna, N., Thliveris, A.T., Ennis, D.G., and Mount, D.W. (1988). Derepression of specific genes promotes DNA repair and mutagenesis in *Escherichia coli*. J. Bacteriol. **170**: 1-4.

Pinkerton, T., Muzyczka, N., Walia, S., Dunn, G., Rose, K., and Duckworth, D. (1981). Bacteriophage T5 growth in *Escherichia coli* containing cloned *Pst*I fragments of the colicin Ib plasmid. Gene **16**: 89-96.

Phizicky, E.M., and Roberts, J.W. (1981). Induction of SOS functions: Regulation proteolytic activity of *E. coli* RecA protein by interaction with DNA and nucleotide triphosphate. Cell **25**: 259-267.

Reed, K.C., and Mann, D.A. (1985). Rapid transfer of DNA from agarose gels to nylon membranes. Nucl. Acids Res. **13**: 7207-7221.

Rees, C.E.D., Bradley, D.E., and Wilkins, B.M. (1987). Organisation and regulation of the conjugation genes of IncI1 plasmid ColIb-P9. Plasmid **18**: 223-236.

Rees, C.E.D., and Wilkins, B.M. (1989). Transfer of tra proteins into the recipient cell during conjugation mediated by plasmid ColIb-P9. J. Bacteriol. **171**: 3152-3157.

Rees, C.E.D., and Wilkins, B.M. (1990). Protein transfer into the recipient cell during bacterial conjugation: studies with F and RP4. Mol. Microbiol. **4**: 1199-1205.

Roberts, J.W., and Roberts, C.W. (1975). Proteolytic cleavage of bacteriophage lambda repressor in induction. Proc. Natl. Acad. Sci. U.S.A. **72**: 147-151.

Roberts, J.W., Roberts, C.W., and Mount, D.W. (1977). Inactivation and proteolytic cleavage of phage lambda repressor *in vitro* in an ATP-dependent reaction. Proc. Natl. Acad. Sci. U.S.A. 74: 2283-2287.

Roberts, J.W., Roberts, C.W., and Craig, N.L. (1978). *Escherichia coli recA* gene product inactivates phage lambda repressor. Proc. Natl. Acad. Sci. U.S.A. 75: 4714-4718.

Roberts, J.W., Phizicky, E.M., Burbee, D.G., Roberts, C.W., and Moreau, P.L. (1982). A brief consideration of the SOS inducing signal. Biochemie 64: 805-807.

Roberts, J. W., and Devoret, R. (1983). Lysogenic induction. In Lambda II. (R.W Hendrix, F.W. Stahl, and R.A. Weisberg. eds). Cold Spring Harbor Laboratory. Cold Spring. Harbor, NY. pp. 123-144.

Rupp, W.D., and Howard-Flanders, P. (1968). Discontinuities in the DNA synthesized in an excision-defective strain of *Escherichia coli* following ultraviolet irradiation. J. Mol. Biol. **31**: 291-304.

Rupp, W.D., and Ihler, G. (1968). Strand selection during bacterial mating. Cold Spring Harbor Symp. Quant. Biol. 33: 647-650.

Ryang Kim, S., and Komano, T. (1989). Cloning and nucleotide sequence of the ColIb shufflon. Plasmid **22**: 180-184.

Salles, B., Paoletti, C., and Villani, G. (1983). Lack of single-stranded DNA-binding protein amplification under conditions of SOS induction in *E. coli*. Mol. Gen. Genet. **189**: 175-177.

Salles, B., and Defais, M. (1984). Signal of induction of recA protein in *E. coli*. Mutat. Res. **131**: 53-59.

Sancar, A., Williams, K.R., Chase, J.W., and Rupp, W.D. (1981). Sequences of the *ssb* gene and protein. Proc. Natl. Acad. Sci. U.S.A. **78**: 4274-4278.

Sancar, A., and Rupp, W.D. (1983). A novel repair enzyme: UVRABC excision nuclease of *Escherichia coli* cuts a DNA strand on both sides of the damaged region. Cell **33**: 249-260.

Sancar, A., Sancar, G.B., Dean Rupp, W., Little, J.W., and Mount, D.W. (1982). LexA protein inhibits transcription of the *E.coli uvrA* gene in vitro. Nature **298**: 96-98.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. **74**: 5463-5467.

Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H., and Roe, B.A. (1980). Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. **143**: 161-178.

Sassanfar, M., and Roberts, J.W. (1990). Nature of the SOS-inducing signal in *Escherichia coli*. The involvement of DNA replication. J. Mol. Biol. **212**: 79-96.

Schneider, R.J., and Wetmur, J.G. (1982). Kinetics of transfer of *Escherichia coli* single strand deoxyribonucleic acid binding protein between single-stranded deoxyribonucleic acid molecules. Biochemistry **21**: 608-615.

Schuster, H., Beyersmann, D., Mikolajezyk, M., and Schlicht, M. (1973). Prophage induction by high temperature in thermosensitive *dna* mutants lysogenic for bacteriophage lambda. J. Virol. **11**: 879-885.

Sedgwick, S.G., and Goodwin, P.A. (1985). Differences in mutagenic and recombinational DNA repair in Enterobacteria. Proc. Natl. Acad. Sci. U.S.A **82**: 4172-4176.

Sedgwick, S.G., Thomas, S.M., Hughes, V.M., Lodwick, D., and Strike, P. (1989). Mutagenic DNA repair genes on plasmids from the 'pre-antibiotic era'. Mol. Gen. Genet. **218**: 323-329.

Sevastopoulos, C.G., Wehr, C.T., and Glasser, D.A. (1977). Large-scale automated isolation of *Escherichia coli* mutants with thermosensitive DNA replication. Proc. Natl. Acad. Sci. U.S.A. **74**: 3485-3489.

Shanabruch, W.G., and Walker, G.C. (1980). Localization of the plasmid (pKM101) gene(s) involved in recA$^+$/lexA$^+$-dependent mutagenesis. Mol. Gen. Genet. **179**: 289-297.

Shinagawa, H., Kato, T., Ise, T., Makino, K., and Nakata, A. (1983). Cloning and characterization of the *umu* operon responsible for inducible mutagenesis in *Escherichia coli*. Gene **23**: 167-174.

Shinagawa, H., Iwasaki, H., Kato, T., and Nakata, A. (1988). RecA protein dependent cleavage of UmuD protein and SOS mutagenesis. Proc. Natl. Acad. Sci. U.S.A. **85**: 1806-1810.

Silhavy, T.J., Berman, M.L., and Enquist, L.W. (1984). in Experiments with Gene Fusions. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, pp. xi-xii.

Simons, R.W., Hoopes, B.C., McClure, W.R., and Kleckner, N. (1983). Three promoters near the termini of IS10: pIN, pOUT, and pIII. Cell **34**: 673-682.

Simonsen, L. (1990). Dynamics of plasmid transfer on surfaces. J. Gen. Microbiol. **136**: 1001-1007.

Slilaty, S.N., and Little, J.W. (1987). Lysine-156 and serine-119 are required for LexA repressor cleavage: A possible mechanism. Proc. Natl. Acad. Sci. USA. 84: 3987-3991.

Soberon, X., Covarrubias, L., and Bolivar, F. (1980). Construction and characterisation of new cloning vehicles. IV. Deletion derivatives of pBR322 and pBR325. Gene 9: 287-305.

Southern, E. (1980). Gel electrophoresis of restriction fragments. Methods in Enzymology 68: 152-176.

Stewart, G.S.A.B., Lubinsky-Mink, S., Jackson, C.G., Cassel, A., and Kuhn, J. (1986) pHG165: A pBR328 copy number derivative of pUC8 for cloning and expression. Plasmid **15**: 172-181.

Strike, P., and Lodwick, D. (1987). Plasmid genes affecting DNA repair and mutation. J. Cell Sci. 6 (suppl): 303-321.

Strobel, M., and Nomura, M. (1966). Restriction of the growth of bacteriophage BF23 by a colicin I (ColIb-P9) factor. Virology 28: 763-765.

Sugino, A., Peebles, C.L., Kreuzer, K.N., and Cozzarelli, N.R. (1977). Mechanism of action of nalidixic acid. Purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. Proc. Natl. Acad. Sci. U.S.A. **74**: 4767-4771.

Thomas, C.M., and Smith, C.A. (1987). Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. Ann. Rev. Microbiol. **41**: 77-101.

Thompson, R., Taylor, L., Kelly, K., Everett, R., and Willetts, N. (1984). The F plasmid origin of transfer: DNA sequence of wild-type and mutant origins and location of origin-specific nicks. EMBO J. 3: 1175-1180.

Thompson, T.L., Centola, M.B., and Deonier, R.C. (1989). Location of the nick site at *oriT* of the F plasmid. J. Mol. Biol. **207**: 505:512.

Traxler, B.A., and Minkley, E.G. Jr. (1987). Revised genetic map of the distal end of the F transfer operon: Implications for DNA helicase I, nicking at *oriT*, and conjugal DNA transport. J. Bacteriol. **169**: 3251-3259.

Tsai, M-M., Fu, Y. -H.F., and Deonier, R.C. (1990). Intrinsic bends and integration host factor binding at F plasmid *oriT*. J. Bacteriol. **172**: 4603-4609.

Tsang, S., Muniyappa, K., Azhderian, E., Gonda, D.K., Radding, C.M., Flory, J., and Chase, J.W. (1985). Intermediates in homologous pairing promoted by recA protein. Isolation and characterization of active presynaptic complexes. J. Mol. Biol. **185**: 295-309.

Ubben, D., and Schmitt, R. (1986). Tn*1721* derivatives for transposon mutagenesis, restriction mapping and nucleotide sequence analysis. Gene **41**: 145-152.

Uemura, H., and Mizobuchi, K. (1982). Inhibition of growth of bacteriophage BF23 by the ColIb plasmid: Identification of the *ibfA* and *ibfB* genes of the ColIb plasmid. Mol. Gen. Genet. **185**: 13-20.

Vapnek, D., and Rupp, W.D. (1970). Asymmetric segregation of the complementary sex-factor DNA strands during conjugation in *Escherichia coli*. J. Mol. Biol. **53**: 287-303.

Varley, J.M., and Boulnois, G.J. (1984). Analysis of a cloned colicin Ib gene: complete nucleotide sequence and implications for regulation of expression. Nucl. Acids Res. **12**: 6727-6739.

Vieira, J., and Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**: 259-268.

Villani, G., Pierre, A., and Salles, B. (1984). Quantification of SSB protein in *E. coli* and its variation during RecA protein induction. Biochemie. **66**: 471-476.

Walker, G.C. (1977). Plasmid (pKM101)-mediated enhancement of repair and mutagenesis: dependence on chromosomal genes in *Escherichia coli* K-12. Mol. Gen. Genet. **152**: 93-103.

Walker, G.C., and Dobson, P.P. (1979). Mutagenesis and repair deficiencies of *Escherichia coli umuC* mutants are suppressed by the plasmid pKM101. Mol. Gen. Genet. **172**: 17-24.

Walker, G.C. (1984). Mutagenesis and inducible responses to deoxyribonucleic acid damage in Escherichia coli. Microbiol. Rev. **48**: 60-93.

Walker, G.C. (1985). Inducible DNA repair systems. Ann. Rev. Biochem **54**: 425-457.

Walker, G. (1987). The SOS response of *Escherichia coli*. In *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., and Umbarger, H.E. (eds). Washington, D.C: American Society for Microbiology, pp. 1346-1357.

Watanabe, T. (1963). Episome-mediated transfer of drug resistance in *Enterobacteriaceae* VI. High frequency transfer system in *Escherichia coli*. J. Bacteriol. **85**: 788-794.

Weaver, C.A., Kagan, B.L., Finkelstein, A., and Konisky, J. (1981a). Mode of action of colicin Ib. Formation of ion-permeable membrane channels. Biochem. Biophys. Acta. **645**: 137-142.

Weaver, C.A., Redborg, H., and Konisky, J. (1981b). Plasmid-determined immunity of *Escherichia coli* K-12 to colicin Ia is mediated by a plasmid-encoded membrane protein. J. Bacteriol. **148**: 817-828.

Weinstock, G.M., and McEntee, K. (1981). RecA protein-dependent proteolysis of bacteriophage λ repressor. Characterization of the reaction and stimulation by DNA-binding proteins. J. Biol. Chem. 256: 10883-10888.

West, S.C., Cassuto, E., and Howard-Flanders, P. (1982). Role of SSB protein in RecA promoted branch migration reactions. Mol. Gen. Genet. **186**: 333-338.

Whittier, R.F., and Chase, J.W. (1981). DNA repair in *E.coli* strains deficient in single-stranded DNA binding protein. Mol. Gen. Genet. **183**: 341-347.

Whittier, R.F., and Chase, J.W. (1983). DNA repair properties of *Escherichia coli tif-1, recAo281 and lexA1* strains deficient in single-strand DNA binding protein. Mol. Gen. Genet. **190**: 101:111.

Wilkins, B.M., Hollom, S.E., and Rupp, W.D. (1971). Deoxyribonucleic acid transferred from ultraviolet-irradiated excision-defective Hfr cells of *Escherichia coli* K-12. J. Bacteriol. **107**: 505-512.

Wilkins, B.M. (1975). Partial suppression of the phenotype of *Escherichia coli* K-12 *dnaG* mutants by some I-like conjugative plasmids. J. Bacteriol. **122**: 899-904.

Wilkins, B.M., Boulnois, G.J., and Lanka, E. (1981). A plasmid DNA primase active in discontinuous bacterial replication. Nature **290**: 217-221.

Willetts, N. (1974). The kinetics of inhibition of F*lac* transfer by R100 in *E. coli*. Mol. Gen. Genet. **129**: 123-130.

Willetts, N. (1977). The transcriptional control of fertility in F-like plasmids. J. Mol. Biol. **112**: 141-148.

Willetts, N., and Achtman, M. (1972). Genetic analysis of transfer by the *Escherichia coli* sex factor F, using P1 transductional complementation. J. Bacteriol. **110**: 843-851.

Willetts, N., and Skurray, R. (1980). The conjugation system of F-like plasmids. Ann. Rev. Genet. **14**: 41-76.

Willetts, N., and Skurray, R. (1987). Structure and function of the F factor and mechanism of conjugation, in: *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, (F. C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, and H.E. Umbarger, eds), American Society for Microbiology, Washington D.C., pp. 1110-1133.

Willetts, N., and Wilkins, B. M. (1984). Processing of plasmid DNA during bacterial conjugation. Microbiol. Rev. **48**: 24-41.

Williams, K.R., Spicer, E.K., LoPresti, M.B., Guggenheimer, R.A., and Chase, J.W. (1983). Limited proteolysis studies on the *Escherichia coli* single-stranded DNA binding protein. J. Biol. Chem. **258**: 3346-3355.

Winans, S.C., and Walker, G.C. (1983). Genetic localisation and characterisation of a pKM101-coded endonuclease. J. Bacteriol. **154**: 1117-1125.

Winans, S.C., and Walker, G.C. (1985). Conjugal transfer system of the IncN plasmid pKM101. J. Bacteriol. **161**: 402-410.

Witkin, E.M. (1976). Ultraviolet mutagenesis and inducible deoxyribonucleic acid repair in *Escherichia coli*. Bacteriol. Rev. **40**: 869-707.

Witkin, E.M., and Kogoma, T. (1984). Involvement of the activated form of RecA protein in SOS mutagenesis and stable DNA replication in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **81**: 7539-7543.

Witkin, E.M., Roegner-Maniscalco, R., Sweasy, J.B., and McCall, J.O. (1987). Recovery from ultraviolet light-induced inhibition of DNA synthesis requires *umuDC* products in *recA718* mutant strains but not in *recA*$^+$ strains of *Escherichia coli*. Proc. Natl. Acad. Sci. USA. **84**: 6805-6809.

Womble, D.D., and Rownd, R.H. (1986). Regulation of IncFII plasmid DNA replication. A quantitative model for control of plasmid NR1 replication in the bacterial cell division cycle. J. Mol. Biol. **192**: 529-548.

Womble, D.D., and Rownd, R.H. (1988). Genetic and physical map of plasmid NR1: comparison with other IncFII antibiotic resistance plasmids. Microbiol. Rev. **52**: 433-451.