The Single-Stranded DNA-Binding Protein Gene of Plasmid ColIb-P9.

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1989
To my parents.
Acknowledgements.

I would like to thank both of my supervisors, Brian for guiding the course of the project and for his helpful advice and criticism, and Peter for his help throughout the project, particularly in the nucleotide sequencing, as well as for his hospitality at Runcorn. Special mention should go to Cath Rees who not only provided the cosmid library and the initial recombinant carrying the ssb gene, but also often preserved the sanity of the lab on the brink of chaos. Thanks all round to Nigel, Angela and Louise, who have all contributed in some way to this work, and without whom the lab would not have been the same! Similar sentiments go to Lyn and Patrick, amongst others, who made life in Runcorn all the more bearable, and the nucleotide sequencing possible. Thanks also to all those, too numerous to mention, who have made the last few years in the Genetics Department both valuable and entertaining. Thanks to John, Karl and Andy in particular, and others who will remain anonymous, for their friendship and exuberance.
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ABBREVIATIONS USED:

A₄₂₀  absorbance at 420 nm
A₅₅₀  absorbance at 550 nm
A₆₅₀  absorbance at 600 nm
bp    base pairs
BSA   bovine serum albumin
cfu   colony forming units
Cmᵣ   chloramphenicol resistance
Col₁ᵇ  colicin Ib resistance
dATP  deoxyadenosine triphosphate
dCTP  deoxycytidine triphosphate
dGTP  deoxyguanosine triphosphate
dTTP  deoxycytidine triphosphate
ddATP dideoxyadenosine triphosphate
ddCTP dideoxycytidine triphosphate
ddGTP dideoxyguanosine triphosphate
ddTTP dideoxythymidine triphosphate
drd    derepressed for transfer
EDTA  sodium ethylene-diamine-tetraacetic acid
HEPES 4-[2-hydroxyethyl]-1-piperazineethanesulphonic acid
Inc   incompatibility group
kb    kilobase
Kmᵣ   kanamycin resistance
λᵣ    resistance to phage λ
min   minutes
Nalᵣ  nalidixic acid resistance
ONPG  ortho-nitryl-phenyl-galactoside
PEG   polyethylene glycol
SDS   sodium dodecyl sulphate
SSG   salts-glucose-casamino acids medium
SSB   single-stranded DNA-binding protein
SSC   salt-sodium-citrate
ssUNA single-stranded deoxyribonucleic acid
<table>
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<th>Abbreviation</th>
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<tr>
<td>TAE</td>
<td>tris-acetate EDTA buffer</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate EDTA buffer</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>Tcr</td>
<td>tetracycline resistance</td>
</tr>
<tr>
<td>TE</td>
<td>tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamide</td>
</tr>
<tr>
<td>TES</td>
<td>Tris EDTA salt buffer</td>
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Single-stranded DNA-binding proteins (SSB) are a specific class of proteins which have a high affinity for single-stranded DNA (ssDNA) to which they bind with no sequence specificity (Chase and Williams, 1986). SSB proteins from a variety of organisms, including *Escherichia coli*, have been shown to play a central role in DNA metabolic processes, including replication, recombination and repair. As discussed later, the properties of the *E. coli* SSB have been elucidated both by investigation of *in vitro* systems and by genetic and biochemical analysis of *ssb* mutants.

Following the discovery that the F plasmid of *E. coli* carries an *ssb* gene (Kolodkin et al., 1983), Golub and Low (1985) demonstrated that many apparently unrelated conjugative plasmids also carry homologous *ssb* genes. The conservation of these sequences suggested that the plasmid *ssb* genes play an important role in the stable inheritance of such plasmids. The objective of the work described here, which was started in October 1985, was to study these plasmid *ssb* genes in an attempt to understand their function.

The two supervisors of this project, Dr. Brian Wilkins and Dr. Peter Barth, had a long-standing interest in conjugative DNA processing in plasmids of the IncII and IncP incompatibility groups, respectively. Consequently one IncII plasmid and one IncP plasmid, both thought to carry *ssb* sequences, were initially investigated. These were ColIb-P9 (IncII), which had already been extensively studied at Leicester, and RP4 (IncP), which was of considerable interest to ICI plc, who supported this work through the SERC-CASE award scheme.
1.2 Single-stranded DNA-binding proteins.

1.2.1 Introduction

Single-stranded DNA-binding proteins (SSB) participate in a variety of DNA metabolic processes, and are required in stoichiometric, rather than catalytic, amounts with respect to ssDNA. A characteristic property of many such proteins is their ability to bind cooperatively to ssDNA, and often they are able to promote the activity of specific DNA polymerases. Several SSB proteins have been identified, both in prokaryotic and eukaryotic cells. Examples of these proteins are those produced by the *E. coli* phages T4 (Alberts and Frey, 1970), T7, M13 and fd (Alberts et al., 1972; Oey and Knippers, 1972), as well as *E. coli* SSB and the three SSB proteins so far identified in yeast (Jong et al., 1985).

The best studied prokaryotic SSB proteins are the phage T4 gene J2 product (gp32) (Alberts and Frey, 1970) and *E. coli* SSB (Sigal et al., 1972). These proteins do not show any extensive sequence homology but appear to be functionally analogous (Chase and Williams, 1986). Since the work described in this thesis relates to proteins showing considerable homology to the *E. coli* SSB, this section will concentrate primarily on the properties of this protein.

1.2.2 Structural and functional domains of *E. coli* SSB.

*E. coli* SSB binds cooperatively and non-specifically to single-stranded DNA (Schneider and Wetmur, 1982), for which it has an affinity about 1000 times greater than for double-stranded DNA (Molineux et al., 1975; Williams et al., 1983). The native form of *E. coli* SSB is a stable tetramer (Bandyopadhyay and Wu, 1978; Molineux et al., 1974; Ruyechan and Wetmur, 1976; Weiner et al., 1975; Williams et al., 1983) of four identical subunits each with a molecular weight of 18,873.
It is predominantly the tetrameric form of SSB which binds ssDNA (Bandyopadhyay and Wu, 1978; Krauss et al., 1981), and this tetramer carries two oligonucleotide binding sites, only one of which can be occupied when binding is cooperative (Ruyechan and Wetmur, 1976).

The nucleotide and amino acid sequences of the ssb gene and protein have been determined (Sancar et al., 1981). The coding region of ssb consists of 534 base pairs, but the methionine of the initiating codon is not present in SSB, which therefore contains 177 amino acid residues. The secondary structure predicted from the amino acid sequence by the method of Chou and Fasman (1978) suggests that the protein contains several functional domains (Sancar et al., 1981). Partial proteolysis studies have subsequently allowed some of these domains to be more precisely defined (Williams et al., 1983).

The amino-terminal two thirds of the protein (residues 1-115) is predicted to be highly ordered, consisting almost entirely of α-helix and β-pleated sheet structures, and it contains 79% of the charged amino acid residues. This region also probably contains the DNA binding domain, since a proteolytic fragment containing only the first 115 residues binds ssDNA at least as well as native SSB (Williams et al., 1983). By sequence comparison of various single-stranded DNA binding proteins, including gp32 and E. coli SSB, Prasad and Chiu (1987) have identified a common triple-stranded β-sheet motif which may be common to the DNA binding sites of many proteins which bind ssDNA without sequence specificity. By the same method, these workers have also identified five aromatic and four charged residues in these ssDNA-binding proteins which are important for binding activity, and have suggested that the DNA binding domain of E. coli SSB lies between residues 29 and 115. Most of the residues important for subunit interactions within the SSB tetramer and for cooperative binding also
appear to be located within the amino-terminal domain (Williams et al., 1983).

The region containing residues 106 to 165 contains only two charged amino acids, and is predicted to be a random coil with a large number of $\alpha$-bends but very little other secondary structure. All the major proteolytic cleavage sites within the protein occur in this region, indicating that it may be available to interact with other proteins (Chase and Williams, 1986; Williams et al., 1983). The carboxy-terminal region of SSB (residues 166-177) is very similar to the analogous region of the T4 gene 32 product, suggesting that these domains may be functionally analogous. This region of both proteins is very acidic and can be removed by partial proteolysis (Hosoda and Moise, 1978; Williams and Konigsberg, 1978; Williams et al., 1983), and cooperative binding of both proteins results in a conformational change which increases the exposure of these domains to proteolytic cleavage (Williams et al., 1983). The binding activity of gp32 is partially repressed by its carboxy terminus (Moise and Hosoda, 1976), and this may be due to interaction between this negatively charged region and the positively charged amino acids at the DNA binding site (Kowalczykowski et al., 1981; Lonberg et al., 1981). Repression is relieved by proteolytic removal of the carboxy terminus or by interactions with other 14 proteins (Burke et al., 1980). It has been suggested that the E. coli SSB carboxy-terminal region has a similar function and that it may interact with other proteins involved in DNA metabolism (Chase and Williams, 1986).

The two best characterized mutations of the E. coli ssb gene are ssb-1, isolated by Sevastopoulos et al. (1977) and ssb-113 (Greenberg et al., 1974; Johnson, 1979; Meyer et al., 1982a). Both the ssb-1 and ssb-113 mutations confer temperature-sensitive lethality due to the rapid cessation of DNA replication at high temperature, and are also
defective in SOS repair and in recombination processes, as discussed in
the following sections.

It is interesting that the ssb-1 and ssb-113 mutations differ in
the effects of temperature on the expression of their associated
defects. Although the temperature sensitivity of strains carrying
either mutation results from a defect in DNA replication, an ssb-1
mutant strain is relatively normal in all functions at low temperature
(30°C), except for a moderate UV-sensitivity which is due to defects in
recombinational repair processes (Lieberman and Witkin, 1981; Lieberman
and Witkin, 1983; Whittier and Chase, 1981). Other defects, described
in later sections, are conferred by the ssb-1 mutation only at the
restrictive growth temperature (42°C), whereas ssb-113 mutant strains
manifest these deficiencies even at a temperature permissive for growth
(Chase et al., 1984; Lieberman and Witkin, 1983). Additionally,
overproduction of the SSB-1 mutant protein, using a multicopy vector,
is able to restore a near normal phenotype in an ssb-1 mutant strain
(Chase et al., 1983b). The different properties of the two mutant
proteins have been partially elucidated by recent biochemical and
sequencing data, discussed later.

The effects of the ssb-1 mutation, and of increased expression of
the ssb-1 gene product, can be attributed to a defect in the stability
of the SSB tetramer (Williams et al., 1984). The ssb-1 mutation
substitutes a histidine for tyrosine at residue 55, within the region
predicted to contain the DNA binding domain (Williams et al., 1982;
Whittier and Chase, 1980), and this residue is thought to be important
for tetramer formation (Williams et al., 1984). The SSB-1 tetramer has
been shown to be unstable in vitro at 25°C at the normal cellular
concentration of SSB of approximately 0.5mM (Chase and Williams, 1986).
Under these conditions the protein exists as an equilibrium mixture of
monomer and tetramer, and the protein is able to bind ssDNA.
At 44°C a reversible alteration of the protein results in a
dramatic decrease in binding. This is probably due to the failure of
the tetramer to form and the thermal instability of the monomer. The
suppression of this defect by overproduction of SSB-1 (Chase et al.,
1983b) suggests that the equilibrium is then shifted in favour of the
thermally stable tetramer.

The ssb-113 mutant protein binds ssDNA with greater affinity than
the wild type protein (Vales et al., 1980). The ssb-113 mutation
results from the substitution of proline for serine at residue 176, the
penultimate amino acid (Williams et al., 1982). This alteration has
effects paralleling the complete loss of the carboxy-terminal portion
of the protein (Williams et al., 1983), substantiating the hypothesis
that the carboxy terminal region is involved in the regulation of
binding activity.

1.2.3 The role of ssDNA-binding proteins in DNA replication.

Both the T4 gene 32 product and E. coli SSB play a central role in
DNA metabolic processes. The T4 tsP7 mutation in gene 32 results in the
cessation of all T4 DNA replication within two minutes of a shift to
non-permissive temperature (Curtis and Alberts, 1976; Riva et al.,
1970). In vitro, gp32 increases by 5 to 10 fold the rate of synthesis
by the T4 gp43 DNA polymerase on a primed ssDNA template (Huberman et
al., 1971). It is thought that gp32 interacts directly with many
proteins involved in T4 DNA metabolism (Formosa et al., 1983).

Following the discovery of E. coli SSB (Sigal et al., 1972), the
requirement for this protein for DNA replication in several ssDNA phage
replication systems in vitro was reported (Scott et al., 1977; Weiner
et al., 1975; Wickner, 1978). Requirement for the SSB in E. coli DNA
replication is indicated by the temperature sensitive growth phenotype
of the ssb-7 and ssb-113 mutant strains, which is due to an immediate
cessation of DNA synthesis at 42°C (Glassberg et al., 1979; Lieberman and Witkin, 1983; Meyer et al., 1979; Vales et al., 1980).

Requirement for the SSB in E. coli DNA replication is also indicated by the finding that the fidelity of replication is increased by up to ten fold by E. coli SSB (Kunkel et al., 1979). E. coli SSB stimulates E. coli polymerases II and III, possibly by removing regions of secondary structure from ssDNA, thus preventing stalling of the polymerase (Molineux et al., 1974; Sherman and Gefter, 1976; LaDuca et al., 1983). E. coli SSB may interact directly with DNA polymerase II since it forms a specific complex with this enzyme (Molineux and Gefter, 1974). In vitro DNA polymerase II will elongate primed DNA which is coated with SSB (Sherman and Gefter, 1976). However, this polymerase is not essential for E. coli chromosomal replication (Campbell et al., 1972), and its role in vivo is not known (Kornberg, 1980).

In addition to possible interactions with DNA polymerase II, the E. coli protein probably participates in direct interactions with other DNA metabolic proteins. It has been suggested that there is a specific interaction between SSB and the rep gene product, 3'-5' helicase, (Tessman and Peterson, 1982), and with protein n, one of at least seven proteins which constitute the primosome of E. coli (Low et al., 1982).

There are an estimated 300-350 copies of the native tetrameric form of the E. coli SSB protein per cell (Weiner et al., 1975; Cuozzo and Silverman, 1985). This compares with approximately 10,000 copies of gp32 in an infected E. coli cell (Alberts and Frey, 1970). However, when the number of replication forks is taken into account, it is apparent that both proteins would be present in sufficient quantities to cover about 1400 nucleotides of ssDNA per replication fork (Chase and Williams, 1986). Since the dissociation rate of SSB from ssDNA is too slow to keep up with a moving replication fork (Krauss et al.,
1981; Romer et al., 1984) it is suggested that SSB can translocate along ssDNA without completely dissociating (Romer et al., 1984). In addition, cooperatively bound aggregates of SSB may be able to transfer directly between DNA strands (Schneider and Wetmur, 1982).

In the presence of SSB, or the T4 gene 32 product, several RecA-mediated reactions are also promoted. These include recombination processes and the SOS response to DNA damaging agents, which are discussed in the following sections.

1.2.4 The SOS response to DNA damaging agents

The SOS response in *E. coli* is an inducible system which includes a number of genes involved in the repair of damaged DNA and in a mechanism, referred to as SOS processing, which results in an elevated level of mutagenesis following DNA damage (Walker, 1985). Induction of the SOS response also results in filamentous cell growth due to the induced expression of the *sfi* genes, which specifically inhibit cell division (Cole, 1983; Huisman and D'Ari, 1981). The SOS system is induced as a response to DNA damage or impediments to DNA replication and is regulated by the products of the *recA* and *lexA* genes. The LexA protein represses the expression of the SOS genes by binding to sequences, termed LexA binding sites or SOS boxes, located near the promoters of these genes (Little and Mount, 1982). The RecA protein is required for the induction of the SOS response, for which it must be in an activated state. In *vitro* this is achieved by the interaction of RecA, single-stranded DNA and a nucleotide co-factor (Craig and Roberts, 1980; 1981; Phizicky and Roberts, 1981; Roberts et al., 1982). In the activated state RecA is able to induce the cleavage of the LexA repressor of the SOS genes (Little et al., 1981; Horii et al., 1981), as well as the repressors of phage λ, 434 and P22 (Roberts et al., 1978). The cleavage of these repressors 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).

In vivo the inducing signal for SOS induction is probably formed by single-stranded regions of DNA generated by SOS-inducing treatments (Bailone et al., 1985; Chaudhury and Smith, 1985; Craig and Roberts, 1980; Devoret et al., 1988; Phizicky and Roberts, 1981; Walker, 1984; 1985). UV damaged DNA itself is able to cause SOS induction, possibly due to the preferential binding of RecA to the partially unwound duplex at the site of DNA damage (Lu and Echols, 1987). Results obtained from temperature sensitive replication mutants of E. coli suggest that single-stranded DNA at the stalled replication fork may act as an SOS inducing signal (Blanco and Pomes, 1977; Witkin, 1976). Indirect induction of SOS functions occurs after the transfer of a UV-damaged ssDNA molecule such as F, F', P1, M13, λ, or Hfr (George and Devoret, 1971; George et al., 1974; Walker, 1984). Although SOS induction by such molecules does not require replication, a more powerful inducing signal results from attempts to replicate the damaged template (D'Arri and Huisman, 1982). The action of SOS repair mechanisms eliminates the SOS inducing signal, reducing the level of RecA activation. The level of LexA will then increase until maximal repression of SOS is again achieved (Little, 1983).

A number of genes encoding products involved in DNA repair processes are expressed by the SOS system. The products of the SOS-inducible uvrA, uvrB, and uvrD genes are required for the excision repair process (Kenyon and Walker, 1981; Kumura and Sekiguchi, 1984; Schendel et al., 1982, van Sluis et al., 1983; Walker, 1985). This process allows the repair of lesions in one strand of a DNA duplex by excision of a fragment containing the lesion and resynthesis of a replacement fragment using the complementary strand as template.
Recombination repair processes, including repair of daughter-strand gaps and double-strand breaks, are also under SOS control. The recA product, itself regulated by the LexA repressor, plays a major role in such processes. In addition to RecA, certain other recombination genes such as recH and ruv are part of the SOS regulon (Walker, 1985).

An additional inducible activity termed SOS processing or error-prone repair accounts for the increased mutagenesis associated with the SOS system and requires the activity of three SOS-inducible genes, recA, umuD and umuC (Elledge and Walker, 1983a). umuDC mutants are non-mutable by and slightly sensitive to UV light and a variety of other chemical agents (Kato and Shinoura, 1977; Walker, 1985; Witkin, 1976), although these strains are not as sensitive as uvr mutants (Walker, 1985). As well as being required for SOS induction, RecA protein has been shown to play additional roles in SOS processing (see below), since lexA recA mutant strains are non-mutable by UV even though umuD and umuC are expressed constitutively in the absence of functional LexA (Blanco et al., 1982; Ennis et al., 1985; Little and Mount, 1982).

A range of naturally occurring plasmids are also able to increase the mutability of E. coli cells (Walker, 1984) and to suppress the effects of umuDC mutations. These plasmids carry SOS-inducible analogues of umuD and umuC (Elledge and Walker, 1983b). Plasmid pKM101, a derivative of R46 (IncN) carries two genes termed mucA and mucB (Walker, 1977; 1978; 1979) while TP110 and ColIB-P9 (IncII) possess the imp genes (I group mutation and protection genes; Dowden et al., 1982; 1984; Dowden and Strike, 1982; Howarth, S., 1965, 1966; Kopylov et al., 1984; Perry and Walker, 1982; Strike and Lodwick, 1987). In addition, investigations using the pR plasmid, a derivative of R46, suggest that plasmids carrying umuDC analogues may also possess
genes whose products are able to antagonize the LexA repressor and therefore positively regulate SOS functions (Battaglia et al., 1987; Gigliani et al., 1981). However, the overproduction of the MucAB proteins has an inhibitory effect on SOS induction by UV, mitomycin C or nalidixic acid (Marsh and Walker, 1987).

The sequence of the umu and muc genes has been determined (Perry et al., 1985). Both sets of genes are located within an operon and share approximately 50% homology. In addition, umuD and mucA share approximately 30% homology with the carboxy terminal region of LexA protein and the λ, 434 and P22 repressors (Perry et al., 1985). This homology includes the region corresponding to the LexA and λ cl cleavage sites (Perry et al., 1985), suggesting that the UmuD and MucA proteins may interact with RecA in a similar way to LexA. The imp genes are also part of an operon, expressing two protein products, and thus show at least some similarity to both the umuDC and mucAB genes (Glazebrook et al., 1986; Strike and Lodwick, 1987).

Recently, overproduction of UmuD from a recombinant plasmid has allowed the protein to be purified. The protein has been shown to be capable of autodigestion at alkaline pH, conditions similar to those required for LexA and λ cl autodigestion, and to undergo a proteolytic cleavage mediated by activated RecA protein (Burckhardt et al., 1988). In vivo, RecA protein-dependent cleavage of the 17 kDa UmuD protein has been observed and the resulting 14 kDa truncate detected. The size of this truncate is in agreement with that expected if UmuD were cleaved at the Cys-Gly bond in the region homologous to that containing the Ala-Gly cleavage site of LexA and λ cl (Shinagawa et al., 1988). Furthermore, Nohmi et al. (1988), have demonstrated that an engineered UmuD, lacking the small amino-terminal fragment normally removed by this cleavage reaction, is sufficient for UmuD mediated mutagenesis. The failure of this truncate to suppress the non-mutability of a lexA
recA mutant strain suggests that RecA plays a further role in SOS processing, in addition to SOS induction and activation of the UmuD protein (Nohmi et al., 1988).

The exact mechanism by which UmuD and UmuC or their analogues generate mutations is unclear but it probably involves specific interactions between the two proteins, since strains carrying combinations of one of each of the muc and umu genes are non-mutable by UV and the gene products are therefore not interchangeable (Nohmi et al., 1988; Perry et al., 1985). Increased mutagenesis possibly results from a reduction in the fidelity of DNA synthesis due to both inhibition of the 3'-5' proofreading exonuclease of DNA polymerase III, an inhibition which may be achieved by RecA protein itself (Fersht and Knill-Jones, 1983; Lu et al., 1986), and the capacity to promote elongation past lesions, which may be the step mediated by the Umu or Muc proteins (Bridges and Woodgate, 1985; Caillet-Fauguet and Maenhaut-Michel, 1988). Although this system allows continued DNA replication past lesions, other repair mechanisms are capable of repairing DNA damage with much greater fidelity, and it is possible that the primary role of the umuDC genes and their plasmid-encoded analogues may be to cause mutagenesis repair itself. This might act to increase genetic variability under conditions of environmental stress (Echols, 1981).

The SOS response in E. coli requires the activity of an SSB protein, and the role of E. coli SSB in RecA promoted reactions, including the SOS response, is discussed in the following section.
The interaction of *E. coli* SSB, ssDNA and RecA protein

In addition to its role in DNA replication processes, the *E. coli* SSB protein also promotes several RecA-mediated reactions. Both *ssb*-I and *ssb-113* strains show increased UV-sensitivity (Lieberman and Witkin, 1983; Whittier and Chase, 1983) and defects in induced RecA protein synthesis (Baluch *et al.*, 1980; Meyer *et al.*, 1982b) and *λ* prophage induction (Cohen *et al.*, 1983; Resnick and Sussman, 1982a; Vales *et al.*, 1980). The increased UV sensitivity of an *ssb uvr* double mutant, compared to an *ssb* mutant, indicates that SSB does not play a major role in excision repair (Whittier and Chase, 1981), an SOS-induced repair process. However, an *ssb recA* double mutant does not show such an increase in sensitivity, suggesting that SSB is required for repair processes which are directly mediated by RecA (Whittier and Chase, 1981). These observations suggest that SSB has an involvement in the SOS response to DNA damage.

The coprotease activity of RecA, required for the cleavage of the repressor of the SOS repair system (*LexA*) and phage *λ* repressor, is promoted by SSB (Little *et al.*, 1980; Moreau and Roberts, 1984; Resnick and Sussman, 1982; Roberts *et al.*, 1978), while SSB prevents the inhibition of phage *λ* repressor by excess ssDNA (Craig and Roberts, 1980; Resnick and Sussman, 1982). The proteolytic activity of RecA under conditions of excess ssDNA is also stimulated by SSB (Weinstock and McEntee, 1981). However, the results of Resnick and Sussman differ from those of Phizicky and Roberts (1980), who found no significant effect of SSB on the repressor cleavage reaction, and from those of Weinstock and McEntee (1981), who found that larger than optimal concentrations of SSB inhibited the cleavage reaction. These conflicting results may result from a different order of addition of reaction components, since when SSB is added before either ATP or RecA protein a stable SSB:ssDNA complex is formed which effectively blocks
the binding of RecA (Morrical et al., 1986, Kowalczykowski and Krupp, 1987).

The ssb-I and ssb-113 strains of E. coli also show defects in recombination (Glassberg et al., 1979; Golub and Low, 1983), and SSB has been shown to markedly increase the rate and extent of the RecA-promoted strand assimilation reaction (Cassuto et al., 1980; Cox and Lehman, 1982; Cox et al., 1983; Egner et al., 1987; McEntee et al., 1980; Shibata et al., 1980; West et al., 1982). It also reduces the amount of RecA required for this process and prevents the inhibition of D-loop formation by excess DNA (Chase, 1984). SSB possibly promotes the recombination reaction by removing secondary structure from ssDNA, eliminating an impedement to RecA protein binding (Muniyappa et al., 1984; Tsang et al., 1985).

Flory and Radding (1982) have made electron microscopic observations which indicate that SSB increases by more than fifty-fold the formation of extended filaments of ssDNA and RecA protein, thought to be the active complex required for strand exchange reactions, while blocking the formation of aggregates of collapsed fibres of ssDNA and RecA. Recently, however, it has been suggested that both E. coli SSB and the T4 gene 32 product may accelerate the forward rate of strand exchange by inhibiting the reinitiation of strand invasion by the displaced single strand, thus preventing the reversal of the reaction (Chow et al., 1988).

The beaded appearance of complexes of ssDNA and SSB at low SSB to nucleic acid ratios, seen by electron microscopy (Sigal et al., 1972; Chrysogelos and Griffith, 1982; Griffith et al., 1984), suggests that SSB organizes ssDNA into nucleosome-like units and the interaction of ssDNA with SSB probably involves the winding of the ssDNA around a core of one or two tetramers of SSB (Chrysogelos and Griffith, 1982; Krauss et al., 1981). Micrococcal nuclease digestion of ssDNA:SSB complexes
indicated that the nucleoprotein chain consists of a core of eight molecules of SSB which are bound to 145 bases of ssDNA. These nucleosome-like structures are linked by approximately 30 base pairs of protein-free DNA which might be available to interact with other proteins, such as RecA (Chrysocholos and Griffith, 1982; Griffith et al., 1984).

However, further in vitro observations have shown that at higher SSB to nucleic acid ratios, a smooth, unbeaded nucleoprotein filament is apparent (Griffith et al., 1984). Therefore SSB apparently has two binding modes, which probably correspond to the detected ssDNA binding site sizes of 33 bases, for the smooth filament, and 65 bases, for the beaded structure, respectively (Bobst et al., 1985; Chase and Williams, 1986; Lohman and Overman, 1985). The transition between the two binding modes depends both on the relative concentrations of ssDNA and SSB and on NaCl concentrations which lie within the physiological range. The larger binding site size is prominent at NaCl concentrations above 0.2 M, whereas the 33 base binding site is prominent below 0.01 M NaCl. A mixture of these two binding modes exists at intermediate NaCl concentrations (Chase and Williams, 1986).

From studies of the rate of dissociation of RecA from ssDNA in the presence and absence of SSB it was inferred that a direct protein:protein interaction between SSB and RecA increased the stability of the RecA:ssDNA complex (Cox et al., 1983; Cox and Lehman, 1982). Munyappa et al. (1984) demonstrated that secondary structure within single-stranded DNA inhibits the association of RecA protein with the DNA, inhibiting RecA-mediated reactions. It was therefore proposed that the function of SSB protein was to remove this secondary structure, allowing RecA to bind to these previously inaccessible regions (Kahn and Radding, 1984; Munyappa et al., 1984). Direct binding studies have suggested that SSB interacts directly with
RecA:ssDNA complexes to stabilize the interaction of RecA and ssDNA and it may be a complex of SSB, RecA and ssDNA which is required for the elimination of secondary structure (Morrical et al., 1986).

Kowalczykowski and Krupp (1987) have characterized the effect of SSB protein on the single-stranded DNA-dependent ATPase activity of the RecA protein, which is associated with the strand assimilation reaction in homologous DNA recombination (Radding, 1982). It was found that SSB can either inhibit or stimulate this activity, depending on experimental conditions. SSB inhibits the ATPase reaction when it is added to ssDNA before RecA protein is added or when ssDNA devoid of secondary structure is used as substrate. However, when secondary structure is present in the ssDNA or the DNA is allowed to renature in the presence of RecA protein, no inhibition is detected. SSB was unable to inhibit completely the ATPase activity of RecA. In addition, a tenfold stabilization of a RecA:ssDNA complex by SSB has been observed, but only under conditions where the ssDNA substrate contained regions of secondary structure (Kowalczykowski et al., 1987). Thus SSB appears only to be required for stabilization of the RecA:ssDNA complex when secondary structure is present in the DNA.

It has been suggested that RecA protein may initially only be able to bind to regions of ssDNA devoid of secondary structure (Kowalczykowski and Krupp, 1987). The binding of SSB may then result in the elimination of regions of secondary structure allowing the formation of an intermediate structure in which RecA and SSB are bound to the single strand. The failure of RecA to destabilize regions of secondary structure is consistent with its inability to destabilize duplex DNA (Cazenave et al., 1984). Whether it is the beaded or filamentous structure which is prominent at this stage is unclear and probably depends on the relative concentrations of SSB and ssDNA. Possibly RecA initially binds only to small regions of ssDNA, perhaps in the linker.
regions proposed by Chrysogelos and Griffith (1982). These short tracts of RecA may be active for repressor cleavage (Moreau, 1987), and may initiate the cooperative binding of RecA to form the longer nucleoprotein filaments necessary for the initiation of strand exchange reactions. The final outcome of the binding reaction then depends on the reaction conditions, including the temperature, magnesium ion concentration and the availability of ATP.

Direct binding studies, in which the quenching of the intrinsic tryptophan fluorescence of SSB has been monitored, indicated that the binding of RecA and SSB proteins is always mutually exclusive and therefore competitive (Kowalczykowski et al., 1987). Results from gel filtration and electron microscopic studies have shown that RecA is displaced from ssDNA by SSB (Cohen et al., 1983; Tsang et al., 1985; Register and Griffith, 1985). However, in the presence of ATP and depending on the temperature and magnesium ion concentration, RecA was shown to bind more tightly to ssDNA than SSB. It was therefore suggested that the high-affinity form of RecA induced in the presence of ATP (Menetski and Kowalczykowski, 1985) may be able to displace bound SSB.

Conversely, these workers also suggested that a low affinity ADP-bound form of RecA resulting from hydrolysis of ATP (Menetski and Kowalczykowski, 1985) could be displaced by SSB. Thus the proportion of SSB and RecA bound would be constantly changing as RecA-mediated ATP hydrolysis proceeded. This would be consistent with the stoichiometric rather than catalytic requirement for SSB. Kowalczykowski and Krupp (1987) proposed that RecA and ssDNA may then ultimately be able to form a unique complex, which requires the presence of DNA homology and base pairing, which is resistant to displacement by SSB.

However, Morrical et al. (1986), also using fluorescence quenching studies, detected no displacement of SSB at any concentration of RecA.
in the presence of ATP. These workers also reported that the interaction of SSB with a RecA:ssDNA complex appears to be distinct from the normal binding interactions of SSB with ssDNA, as determined by kinetic properties, since it does not result in the displacement of RecA from the single strand (Morrical et al., 1986). It has been speculated that a direct interaction between RecA and SSB may result in a conformational change in SSB, possibly similar to that induced by low salt concentrations.

The stoichiometry of RecA protein binding to ssDNA is reduced under conditions which favour the formation of ssDNA secondary structure, while the presence of SSB removes this barrier to RecA binding. Since SSB is apparently not displaced by RecA, the removal of secondary structure may be a property of a joint SSB:RecA complex, and it may therefore be the formation of this joint complex of RecA and SSB which accounts for the stoichiometric requirement for SSB (Morrical et al., 1986).

The nature of the structures formed under conditions of excess SSB are unclear and the effects of overproduction of SSB are often apparently contradictory. In vitro experiments by Cohen et al. (1983) demonstrated that, whereas excess SSB was able to inhibit RecA promoted ATPase activity, the binding of SSB cannot completely eliminate binding of RecA to ssDNA. Furthermore, RecA coprotease activity could not be eliminated by SSB. Similarly, whereas overproduction of SSB in vivo inhibits UV induced RecA-dependent recombination processes and results in a mild increase in UV sensitivity, it has only relatively small effects on LexA and λ repressor cleavage (Moreau, 1987; 1988). These results suggest that, whereas the formation of the long nucleoprotein filaments required for the initiation of recombination is inhibited by excess SSB, the formation of the short tracts of RecA required for repressor cleavage is unaffected (Moreau, 1987).
It has been suggested that, in a DNA-limited complex of ssDNA, RecA and SSB, the molecular conformation of the RecA protein is such that the active site for ATP hydrolysis is occluded while that for coprotease activity is exposed. When more sites are available on the ssDNA, a conformational change in RecA might reverse this arrangement (Cohen et al., 1983). A model proposed by Cohen et al. (1983) suggested that even at high SSB concentrations RecA was able to bind to the 30 base pair linker regions present in the beaded structure observed by Chrysogelos and Griffith (1982) and that this explained the inability of excess SSB to inhibit the repressor cleavage reaction. However, the beaded structure is apparent at low SSB to ssDNA ratios, and so may not explain the effects of high levels of SSB (Chrysogelos and Griffith, 1982; Chase and Williams, 1986). However, the structure formed by ssDNA and SSB in vivo is far from clear since it is apparently dependent on many factors. Moreau (1988) has suggested that the effect of excess SSB on recombination processes may be explained by a reduction in the rate of polymerization of RecA on long stretches of ssDNA, as a result of competition for binding sites by excess SSB. However, this competition for binding sites might have only minimal effects on the formation of the short tracts of RecA-bound ssDNA which are thought to be required for coprotease activity.

Although excess SSB greatly inhibits UV-induced recombination processes, there is apparently little effect on spontaneous recombination of undamaged DNA or on SOS mutagenesis processes. This may be explained by the involvement of the RecBC and UmuDC proteins, respectively, which possibly act in concert with RecA in these processes and prevent the binding of SSB (Moreau, 1988).

Another apparent inconsistency is the finding that, after UV irradiation, overproduction of SSB reduces the rate of induction of SOS genes in vivo, despite the partial derepression of these genes by
excess SSB in noninduced bacteria (Moreau, 1988). The partial induction of SOS activities in cells overproducing SSB may be due to impediments in the progression of DNA polymerase caused by binding of SSB at the replication fork. This would increase the availability of single-stranded DNA for RecA activation and hence stimulate LexA cleavage (Moreau, 1987). Possibly the rate of LexA cleavage is reduced in these cells after induction if the longer RecA:ssDNA filaments are prevented from forming by excess SSB (Moreau, 1988; Roberts et al., 1982). Such filaments may normally be active for repressor cleavage prior to their involvement in recombination (Julin et al., 1986; Moreau, 1988).

Obviously there are many aspects of SSB activity which remain unclear. The outcome of reactions which require the binding of SSB to ssDNA apparently depends on many factors, including, among others, the relative concentrations of ssDNA, SSB and RecA and, in _in vitro_ experiments, on the order of addition of these components. The conflicting results obtained by different workers probably reflect differences in the experimental conditions used. The role of the observed ssDNA:SSB structures and their correlation with the cooperative binding of SSB remains obscure. In addition, the processes in which SSB is involved probably also require the activities of other DNA metabolic proteins. For example, it has been suggested that the _recF_ product may interact with RecA to promote the displacement of SSB from ssDNA, since the overproduction of SSB mimics some of the characteristics of _recF_ mutant strains (Moreau, 1988).

The effects of the _ssb_-I and _ssb_-II3 mutations on RecA-promoted reactions may be explained by the affinity of the mutant proteins for ssDNA. Since the SSB-II3 protein binds more strongly to ssDNA than wild type SSB (Vales et al., 1980), the mutant protein may have an enhanced ability to displace RecA from the ssDNA, although SSB-II3 may confer a
different conformation on the ssDNA than does the wild-type protein. The SSB-1 protein has a reduced binding affinity (Williams et al., 1984) and may therefore be less efficient in promoting the removal of secondary structure from the ssDNA prior to RecA binding or may be defective in its association with the RecA:ssDNA complex.

1.2.6 Regulation of E. coli ssb expression.

If SSB plays a major role in SOS processes it might be expected that the ssb gene would be under LexA control. The ssb gene lies adjacent to the inducible uvrA gene (Sancar et al., 1981) and the ssb and uvrA genes are transcribed in opposite directions (Brandsma et al., 1983; Sancar et al., 1981). The -35 sequence of uvrA overlaps a LexA binding site (Hackendorf et al., 1983), with ssb lying within 200 base pairs of the uvrA start point (Sancar et al., 1981). Despite this arrangement and the known involvement of SSB in the SOS response, many workers have failed to detect SOS induction of SSB (Alazard, 1983; Salles et al., 1983; Whittier and Chase, 1981; Villani et al., 1984). However, using fusions between the galK structural gene and ssb, Brandsma et al. (1983) measured an increase in galactokinase levels after DNA damage, and it was suggested that an inducible promoter of ssb, designated PI, was necessary for SOS induction of ssb. Both this promoter and two other non-inducible promoters downstream of PI have been identified by S1 mapping. Transcription from the two non-inducible promoters appears to decrease following SOS induction (Brandsma et al., 1985).

Studies using radioimmunoassays (Paoletti et al., 1982) to quantify the level of SSB failed to detect any amplification of SSB under SOS-inducing conditions (Salles et al., 1983; Villani et al., 1984). This observation is in agreement with results which indicated that, in a non-inducible lexA3 strain, the LexA repressor inhibits uvrA
induction but does not affect the level of \textit{ssb} expression (Alazard, 1983). More recently the rate of SSB synthesis during SOS induction has been reexamined by radioimmunoassay (Perrino \textit{et al.}, 1987). These results indicated that, although an increase in the relative rate of synthesis of SSB was apparent after SOS-inducing treatments, there was no detectable increase in the level of accumulated SSB protein. Moreover, RecA protein was synthesised roughly twenty times faster than SSB. Together these results indicate that the increase in SSB synthesis during SOS induction is not significant with regard to SOS processes and that the three putative promoters may stringently maintain a constant high level of SSB (Perrino \textit{et al.}, 1987).

1.2.7 \textbf{Plasmid-encoded single-stranded DNA-binding proteins.}

Whilst constructing various \textit{E. coli} strains carrying the \textit{ssb-1} mutation, Kolodkin \textit{et al.} (1983) found that the introduction of the \textit{F} sex factor of \textit{E. coli} resulted in a partial suppression of the temperature-sensitive growth phenotype caused by the chromosomal \textit{ssb-1} or \textit{ssb-II3} mutations. An \textit{ssb-1} strain harbouring \textit{F} was able to form colonies at a temperature 2°C higher than the parental \textit{ssb-1} strain. It has been noted, however, that both the growth conditions and the background genotype of the \textit{ssb-1} strain can markedly alter the effect of temperature on cell survival (Lieberman and Wilkin, 1981).

It was demonstrated that the \textit{ssb-1} suppressing effect was due to the expression of a protein product, here designated \textit{F SSB}, which binds tightly to single-stranded DNA (Kolodkin \textit{et al.}, 1983) under conditions similar to those at which the chromosomal SSB binds (Sigal \textit{et al.}, 1972; Molineux \textit{et al.}, 1974). The gene responsible, \textit{F ssb}, was demonstrated to be present within the \textit{EcoRI} fragment \textit{J} (F3) of the \textit{F} factor at approximately 55.2 kilobases on the standard map of \textit{F} (Willetts and Skurray, 1980). This is within the region of \textit{F}.
transferred early during bacterial conjugation (Cram et al., 1984).

The amino acid sequence of F SSB and the nucleotide sequence of the F ssb gene were determined (Chase et al., 1983a). The protein contains 178 amino acids, one more than E. coli SSB, and has a molecular weight of 19,505. The initiating methionine is absent, as in the E. coli protein. F SSB has extensive sequence homology to E. coli SSB, particularly in the amino-terminal region, where 87 of the first 115 amino acid residues are identical. The corresponding portion of the E. coli protein contains the DNA binding domain (see Section 1.2.2).

Although several small regions of homology exist, the two proteins diverge extensively in the carboxy-terminal region. However, six of the last seven amino acids are identical, suggesting that this region is important for the activity of both proteins.

Subsequently the ability of conjugative plasmids from 23 different incompatibility (Inc) groups to suppress defects in the chromosomal ssb gene was investigated (Golub and Low, 1985). It was found that plasmids from 12 of these Inc groups were able to suppress ssb-1 defects. These included plasmids from incompatibility groups FI, FII, FIV, FY, FVI, II, Y, 9, T, B, K and P. Since plasmids from different Inc groups have little sequence homology (Falkow et al., 1974; Grindley et al., 1973), the presence of homologous ssb genes on these apparently distinct plasmids suggested an important role for the plasmid-encoded SSBs.

Genes responsible for ssb-1 suppression were cloned from plasmids chosen from three Inc groups, IncII, IncY and Inc9. These genes were shown by DNA-DNA hybridization techniques to be homologous to the cloned F ssb gene and to each other, as well as to E. coli ssb (Golub and Low, 1985). However, the plasmid genes were judged to be more homologous to each other than to the E. coli gene. Southern hybridization also showed that all conjugative plasmids found to suppress ssb-1 defects carried genes homologous to F ssb, whereas non-
suppressing plasmids carried no similar sequences.

The role of the plasmid-encoded SSB proteins remains unclear. Mini-F plasmids which do not carry the F ssb region were known to have no apparent defect in replication (Lovett and Helinski, 1976; Timmis et al., 1975), at least in an ssb+ host, and it therefore seemed unlikely that the role of these proteins was in plasmid replication.

More recently, and since the commencement of this project, it has been shown that conjugative plasmids which carry DNA homologous to F ssb also have homology to a considerable part of the 12.9 kb leading region of F. This region carries the origin of conjugative transfer (oriT; see Section 1.3.3) at one end and is transferred first during conjugation (Cram et al., 1982; Ray and Skurray, 1983; Willest and Skurray, 1980). This finding suggested that the whole leading region, including ssb, may play an important role in the stable inheritance of the conjugative plasmids showing this homology (Golub and Low, 1986a).

Following the initiation of the work described in this thesis it was demonstrated that plasmids carrying mutations which derepress the genes involved in the conjugative transfer process (see Section 1.3.2) show a higher level of ssb-1 suppression than the parental repressed plasmids, R1, R64 and R222 (Golub and Low, 1986b). It was found that plasmids derepressed for conjugation functions synthesised more RNA which hybridized to a small (0.7 kb) fragment carrying F ssb than did the parental plasmids. Thus it was clear that the plasmid ssb genes were coordinately controlled with the transfer functions. It was reported that no defect in conjugation at 43°C, in conjugative replication processes, or in the ability to promote transfer of small mobilizable plasmids apparently resulted from defects in the ssb genes of F factor or plasmid R483 (IncII), but no data were given (Golub and Low, 1986b). It was also reported that the HfrC (Cavalli) strain of E. coli, when made ssb−, has the same temperature and UV sensitivity.
as an \textit{ssb-1} strain. Thus HfrC does not carry a functional F \textit{ssb} gene. However, the Hfr is very fertile, again indicating that the plasmid \textit{ssb} is not required for conjugative transfer, at least when an active chromosomal \textit{ssb} gene is present. These later results agree with results presented in this thesis with regard to an \textit{ssb} mutant of the \textit{IncI1} plasmid Col\textit{bdrd-1}. 
1.3 Conjugative plasmids and bacterial conjugation.

1.3.1 Introduction.

Conjugative plasmids studied in enterobacteria include members of more than twenty incompatibility groups (Jacob et al., 1977). The first conjugative plasmid to be identified was the E. coli fertility factor, now referred to as F (Cavalli-Sforza et al., 1953; Hayes, 1953). Although many other conjugative plasmids have now been identified, both in gram-positive (Helinski et al., 1985) as well as gram-negative bacteria, the conjugation systems of F and the F-like plasmids are the best characterized (Ip pen-Ihler and Minkley, 1986; Willetts and Skurray, 1987). Plasmid Colll-P9 (IncII), and the related plasmids R64 (IncII) and R144 (IncII+B), encode the II conjugation system, which resembles that of F but differs in a number of specific features (Rees et al., 1987). These two conjugative systems are compared in this section.

1.3.2 The F and II conjugation systems.

The F transfer (tra) genes lie within a 33.3 kb tra region, which is bounded by the origin of transfer (oriT), (Vapnek et al., 1971; Johnson and Willetts, 1983) and an IS3 insertion sequence in the finO gene (Cheah et al., 1986). The location of the genes within this region have been determined and a map of the known tra functions of F has been produced (Ip pen-Ihler and Minkley, 1986; Traxler and Minkley, 1987). The F transfer genes are organized into five main transcriptional units (Manning et al., 1984) with the majority of the genes lying within the large traY-Z operon. Only the traM, traJ and finP genes, described below, are located outside the traY-Z region (Willetts and Skurray, 1987).

Although the traS and traT genes are located together within the
trαY-Z operon, they are not required for transfer itself and may be expressed from weak internal promoters (Cheah et al., 1986). These genes specify products required for surface exclusion, a mechanism which prevents mating between two cells harbouring similar plasmids (Achtman et al., 1977). The trαδ product may act in the inner membrane to prevent DNA transfer, while the trαT product is located in the outer membrane and possibly prevents mating pair formation (Achtman et al., 1977; Willetts and Skurray, 1987). Plasmids of the IncI group similarly express exclusion genes (Hartskeerl et al., 1985; Rees et al., 1987).

As is the case for many conjugative plasmids (Meynell et al., 1968), the transfer systems of most F-like plasmids are subject to negative control, although the effects of such repression are not fully exerted for several hours following transfer (Willetts, 1974; Willetts and Skurray, 1987). In these plasmids, repression is due to the FinOP fertility inhibition system (Meynell et al., 1968). The products of the finO and finP genes act in concert to inhibit expression of the trαJ gene, which is the positive regulator required for the expression of most of the other transfer genes (Cuozzo and Silverman, 1985; Finnegan and Willetts, 1972; Willetts, 1977; Willetts and Skurray, 1980). The finP product is thought to be an RNA molecule carrying complementary sequences to the leading end of the trαJ mRNA and may exert transcriptional or translational control by interactions with trαJ sequences (Willetts and Skurray, 1987). Plasmid F itself is naturally derepressed for expression of the transfer genes due to the disruption of the finO gene sequence by the IS3 insertion (Cheah et al., 1986). Optimal expression of the F transfer functions also requires the activities of several chromosomal genes (Mullineaux and Willetts, 1985; Thompson and Taylor, 1982; Willetts and Skurray, 1987).

It has recently been determined that the ColIlb-P9 conjugation
genes are organized into three separate transfer regions, contained within a 50 kb segment, and that tra gene expression requires the activity of two positive regulators rather than the one product (traJ protein) required for the F system (Rees et al., 1987). As is the case for F-like plasmids, derepressed (drd) mutants of the 11 plasmids can be obtained by the disruption of a negative regulatory system that presumably governs the positively required products (Meynell and Datta, 1967). Unusually, plasmids of the I complex of incompatibility groups (IncI1, B, K, I2) specify two different types of conjugative pilus of thick rigid and thin flexible morphology, distinguishing these plasmids from other conjugative plasmids studied so far (Bradley, 1983; 1984). In ColIb-P9 each type of pilus is encoded by genes from distinct tra regions, each associated with one of the two positive regulators. The thick 11 pili mediate surface-obligatory conjugation and resemble most closely those encoded by IncM plasmids (Rees et al., 1987), while the thin flexible pili show a superficial similarity to those specified by many plasmids, including those of the F-complex. Another feature which distinguishes plasmids specifying the 11 conjugation system from plasmid F is the specification by the former of a DNA primase, which is required for conjugative DNA processing (Wilkins et al., 1981; Chattfield et al., 1982). A few other groups of conjugative plasmids, including those from IncM and IncP groups, also specify a DNA primase (Lanka and Barth, 1981). Thus part of the ColIb conjugation system shows some resemblances to the IncM and IncP systems. The expression of two, rather than one type of conjugative pilus and the requirement for two separate positive regulators suggests that ColIb may be derived from the fusion of parts of two ancestral conjugative plasmids (Rees et al., 1987).
1.3.3. The conjugative transfer process.

Two types of process are involved in the transfer of conjugative plasmids. The first of these involves cell surface interactions, requiring the synthesis of the conjugative pili, which act to contact the recipient cell surface and to bring donor and recipient together. However, extended flexible pili as specified by F-like plasmids are not the normal route of transport for DNA since DNA transfer from an Hfr donor is unaffected by treatment of cells with sodium dodecyl sulphate after mating pair formation (Panicker and Minkley, 1985).

The activities of other plasmid-encoded products, which stabilize aggregates of mating cells and form the as yet undetermined transfer bridge are also necessary (Bradley, 1981). These include the traN and traS proteins (Willetts and Skurray, 1987). However, these surface interactions are not the subject of this work. Instead this section will concentrate on the second process involved in conjugative transfer, the processing of the plasmid DNA itself. For F-like plasmids this requires the activities of the tra genes M, Y, D, and I (Everett and Willetts, 1980; Kingsman and Willetts, 1978).

The initiation of conjugative plasmid DNA transfer occurs at a specific site, the origin of transfer or oriT (Guyer and Clark, 1976; Guyer et al., 1977). Such sites are also carried by mobilizable plasmids, such as ColEl (Bastia, 1978). These mobilizable plasmids can be transferred as independent units when the functions necessary for cell to cell interactions are provided in trans by a suitable conjugative plasmid (Finnegan and Sherratt, 1977; Warren et al., 1978; 1979). In addition to a specific oriT site, mobilizable plasmids also carry mob genes whose products are required for the mobilization of these plasmids (Dougan and Sherratt, 1977; Warren et al., 1978).

The origin of transfer regions from many conjugative plasmids have been cloned and recombinants carrying these sites are also mobilizable.
by the homospecific conjugative plasmid (Willetts and Wilkins, 1984).
The function of the oriT site of F as a single-strand nick site has
been demonstrated by the nicking of λ oriT transducing phage in F tra+
cells (Everett and Willetts, 1980). This nicking was shown to require
the products of traY, traZ and to occur in the strand transferred
during conjugation (Everett and Willetts, 1980; 1982). Nicking and
religation at oriT sites is thought to occur continuously in donor
cells (Everett and Willetts; 1980) and is therefore not the step
triggered by the undefined inducing signal for conjugative DNA
transfer. The F oriT sequence is plasmid specific, as is the traY
component of the traYZ endonuclease which is thought to recognise it
(Everett and Willetts, 1980; Willetts and Skurray, 1987). The F traYZ
product is not required for F-mediated mobilization of non-conjugative
plasmids (Everett and Willetts, 1980; Willetts, 1980; Willetts and
Maule, 1979; Willetts and Wilkins, 1984). Differences between the oriT
sequences may account, in part, for the requirement for the mob gene
products of mobilizable plasmids, since these probably include the
specific endonucleases required for nicking at the specific oriT of the
plasmid (Willetts and Wilkins, 1984).

Following nicking at oriT, the nicked single strand is transferred
from donor to recipient. In the case of F, a specific strand is
transferred and it is known that there is a leading 5' terminus (Ihler
and Rupp, 1969; Ohki and Tomizawa, 1968; Rupp and Ihler, 1968). A
specific strand is also transferred in the case of the IncI1 plasmid
R64 and it may be assumed that ColIb-P9 is similar in this respect
(Willetts and Wilkins, 1984). IncI1 plasmids are probably transferred
unidirectionally (Datta and Barth, 1978; Willetts and Wilkins, 1984),
as is the IncP plasmid RP4 (Al-Doori et al., 1982; Grinter, 1981).
Similarly, mobilization of plasmid ColEl is unidirectional, and a
single strand is probably transferred with a leading 5' terminus (Boyd
and Sherratt, 1986).

Unwinding of the two plasmid DNA strands is probably the function of the F \textit{tra}I gene product, which has been shown to be DNA helicase I (Abdel-Monem \textit{et al.}, 1983). This possibly acts in conjunction with the \textit{E. coli} \textit{rep} gene product, a 3'-5' helicase (Ippen-Ihler and Minkley, 1986), which, interestingly, may have interactions with SSB protein (Tessman and Peterson, 1982). The DNA-dependent ATPase activity of the \textit{tra}I helicase may also act to displace the nicked strand into the recipient if the TraI protein were associated with the membrane (Willetts and Wilkins, 1984; Panicker and Minkley, 1985). This may be achieved by the interaction of TraI with the membrane localized \textit{traD} product, which could possibly form part of a specific pore required for transfer (Ippen-Ihler and Minkley, 1986; Willetts and Skurray, 1987).

It has recently been suggested that the \textit{traZ} product is a translation reinitiation product of the \textit{tra}I gene and that it is a \textit{traY-tra}I protein complex which is responsible both for nicking at \textit{oriT} and for unwinding of the plasmid DNA strands (Traxler and Minkley, 1987; Willetts and Skurray, 1987).

The transfer process is usually associated with the synthesis of a replacement strand in the donor and a complementary strand in the recipient (Willetts and Wilkins, 1984). Studies of \textit{dnaC} mutant strains have indicated that donor cell replacement strand synthesis during F transfer is performed by DNA polymerase III (Kingsman and Willetts, 1978). By analogy with the role of \textit{E. coli} SSB in phage and bacterial DNA replication, and its ability to stimulate DNA polymerase III (Molineux \textit{et al.}, 1974), conjugative DNA metabolism may also require the activity of an SSB protein (Willetts and Skurray, 1987). The \textit{ssb} products of F and ColIb may therefore participate in donor cell DNA synthesis.

In the case of both F and IncII plasmids, \textit{de novo} synthesis of RNA
The primer generating mechanisms utilized for donor cell DNA synthesis on F and Pl plasmids differ. In the case of F two alternative mechanisms may exist, one mediated by the primosome and the other by RNA polymerase (Willetts and Wilkins, 1984). Plasmids specifying the Pl conjugation system encode their own DNA primase, which is discussed below.

Synthesis of a complementary strand on the transferred DNA occurs in the recipient cell following transfer. This process does not require the expression of plasmid genes in the recipient (Hiraga and Saitoh, 1975; Boulnois and Wilkins, 1978). Conjugative synthesis in the recipient again requires DNA polymerase III (Wilkins and Hollom, 1974). In the case of F, primers for complementary strand synthesis are thought to be made by RNA polymerase or the activity of the primosome, acting as alternative processes (Willetts and Wilkins, 1984). Unlike F-like plasmids, Pl11 plasmids specify a DNA primase (sog product) which is required for complementary strand synthesis in the recipient cell (Boulnois and Wilkins, 1979).

In both donor and recipient cells, the ColIb-P9 sog primase acts to generate the short RNA primers active in conjugal DNA synthesis (Wilkins et al., 1981; Chatfield et al., 1982; Lanka and Furst, 1984). The sog locus specifies two overlapping polypeptides of apparent molecular weight 180,000 and 240,000 (Wilkins et al., 1981) and the N-terminus of the larger polypeptide is required for primase activity (Chatfield and Wilkins, 1984; Merryweather et al., 1986a). The sog gene products are supplied by the donor cell (Boulnois and Wilkins, 1979) and both polypeptides are transmitted to the recipient by a conjugation-dependent process, probably as a complex with the transferred single-strand (Chatfield and Wilkins, 1984; Merryweather et
The role of the smaller sog polypeptide is unclear, but it appears to act following the initiation of transfer to promote the efficient transmission of DNA (Merryweather et al., 1986a).

In addition to Incl plasmids, plasmids from a number of other incompatibility groups, including the IncP plasmid RP4, also encode DNA primases. However, although the RP4 pri product acts in bacterial conjugation in an analogous fashion to the ColIb-P9 DNA primase, the proteins differ immunologically (Lanka and Barth, 1981) and are not directly interchangable with respect to the conjugation systems of the two plasmids (Merryweather et al., 1986b).

The finding that plasmid-encoded proteins are transferred from the donor to recipient cell during conjugation suggested that other proteins involved in DNA synthesis in the recipient may also be transferred (Willets and Wilkins, 1984). A candidate for one such protein is the plasmid-encoded SSB. This protein binds to ssDNA, and could therefore be transmitted as a complex with the transferring single-strand, as suggested in the case of the sog products. If transferred it is likely that the plasmid-encoded SSB would act to promote complementary strand synthesis on the transferred DNA.

However, many conjugative plasmids do not carry genes homologous with ssb (Golub and Low, 1985), although they may encode analogues of SSB.

An alternative role for the plasmid SSB may be in the protection of the single strands produced during the conjugation process and possibly the prevention of SOS induction by this single-stranded DNA. Recent reports (Baqdasarian et al., 1980; 1986) have suggested that some conjugative plasmids encode products which result in a plasmid-mediated SOS inhibition or Psi phenotype and plasmid SSB could conceivably have a role in such a process.

Circularization of the transferring plasmid is RecA-independent (Clark, 1967) and does not require the expression of plasmid genes in
the recipient. 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 circularize the plasmid by ligation (Willetts and Wilkins, 1984). The TraYZ endonuclease may participate in this process as a type I topoisomerase (Everett and Willetts, 1980; Willetts and Skurray, 1987).

1.4 Plasmid-mediated SOS inhibition.

Recently, the ability of some conjugative plasmids to inhibit SOS induction has been reported. Bagdasarian et al. (1980) initially identified an activity, specified by plasmid R100.1, which suppresses cell filamentation, recA protein induction and λ prophage induction caused by the recA441 mutation of E. coli. This mutation causes gratuitous expression of SOS functions at 42°C in the presence of adenine (Castellazzi et al., 1972). There is no inhibition of UV or mitomycin C-triggered induction of SOS functions by this plasmid. It was shown more recently that the related plasmid R6-5 expresses a similar activity, which is able to suppress SOS induction mediated by either the recA441 or recA730 mutations (Bagdasarian et al., 1986). The latter mutation results in constitutive expression of SOS functions even at low temperature (Witkin and Koqoma, 1984). R6-5 also suppresses SOS induction caused by nalidixic acid treatment of a recA* cell, indicating that the phenomenon is not a feature of the mutant RecA proteins. The Psi activity of R6-5 prevents the induction of bacterial SOS genes, as determined using fusions between the SOS-inducible sfiA gene and lacZ (Bagdasarian et al., 1986), recA730-promoted mutagenesis, and chromosomal recombination (Bailone et al., 1988). The plasmid-specified SOS inhibition function of R6-5 results from the expression of a 12 kDa protein product from a plasmid locus termed psiB (Bailone et al., 1988). This gene lies within the region of the plasmid.
transferred early during conjugation, near the origin of transfer (Bagdasarian et al., 1986; Bailone et al., 1988).

E. Golub (personal communication, 1988) has recently found that a number of plasmids, including the IncII plasmid R483, have extensive homology to the cloned \( \psi \text{II} \) gene of R6-5. These plasmids all show extensive homology to the leading region of plasmid F and possess \( s\text{sb} \) genes (Golub and Low, 1985; 1986a; see Section 1.2.7). Presumably, therefore, the IncII plasmid ColIB-P9 also carries a \( \psi \) gene. Although F was shown to carry a \( \psi \text{II} \) homologue and a recombinant plasmid overexpressing this gene was able to suppress SOS expression (E. Golub, personal communication), F itself does not confer a \( \psi \text{II}^+ \) phenotype (Bagdasarian et al., 1980).

The \( \psi \text{II} \) gene product does not appear to interact with SOS gene operator sites in an analogous fashion to LexA, since the presence of a recombinant plasmid carrying the cloned \( \psi \) locus of R6-5 did not reduce the level of \( s\text{fIA} \) expression in a \( \text{lexA} \) mutant strain. Neither does Psi activity act to reduce expression of RecA, as detected using \( \text{recA}:\text{lacZ} \) fusion strains (Bagdasarian et al., 1986). The efficiency of the Psi function is dependent on the \( \text{recA} \) allele present, indicating that the phenomenon requires an involvement of RecA rather than LexA. Thus it is most likely that Psi interacts with RecA or inhibits its activation (Bailone et al., 1988).

It has been speculated that the role of the Psi proteins specified by conjugative plasmids may be to allow the transfer of long stretches of single-stranded DNA without generating an SOS signal (Bagdasarian et al., 1986). It is unclear how the Psi activity relates to the positive regulation of SOS genes reported in the case of the pR plasmid (Battaglia et al., 1987), mentioned earlier. However, together these findings suggest that certain conjugative plasmids are able to
influence the expression of cellular SOS functions.

1.5 Aims of this work.

Since the *E. coli* SSB was known to be essential for many aspects of DNA metabolism, it was anticipated when this work began in 1985 that the recently identified plasmid-encoded proteins would also have a vital role, possibly in some aspect of conjugative DNA metabolism. No role had yet been attributed to these proteins. The aim of the work described here was therefore to gain some understanding of the function of the plasmid ssb gene products. The approach eventually used was to construct a mutant defective specifically in the ssb gene carried by plasmid Collb-P9. Hereafter, this gene will be called Collb ssb and its product Collb SSB.

Chapter two describes, as an initial step, the mapping of the Collb ssb gene within Collb-P9 and the construction of an insertional mutation within this gene. Results presented in Chapter Two also show that Collb ssb and the transfer genes are coordinately expressed, suggesting a role for Collb SSB in the conjugation process.

In order to locate precisely the insertion site used for the construction of the Collb ssb mutation, and to assess whether the mutant gene might specify an active truncated protein, the nucleotide sequence of the Collb ssb gene was determined. This is described in Chapter Three.

Since the order in which plasmid genes are transferred during conjugation may affect the interpretation of the roles of these genes, the direction of transfer of Collb was determined. Chapter Four describes a technique which was used to demonstrate that, as in the case of F, the Collb ssb gene is located in the region transferred early during conjugation.

Chapter Five reports the results of analyses designed to assess
whether the Colib ssb mutation causes defects in either the transfer efficiency or the maintenance stability of Colib\(\text{ard-1}\). The possibility that Colib SSb is involved in the regulation of the SOS response was investigated in the experiments described in Chapter Six. These data show that the insertion mutation in the ssb gene of Colib\(\text{ard-1}\) confers a strong \(\text{Psi}^+\) phenotype on the host cell.
Chapter Two

The identification, disruption and mode of regulation of a ColIb-P9 gene specifying a single-stranded DNA-binding protein.

Introduction.

The aim of the work described in this chapter was to confirm that the conjugative plasmids ColIb-P9 and RP4 specify a single-stranded DNA-binding protein and to construct appropriate mutants. A plasmid carrying such a mutation could then form the basis for further investigations into the role of the plasmid SSB. The suppression of the temperature sensitivity of ssb-1 mutant strains of *E. coli* at 44°C by plasmids carrying ssb sequences presented a convenient method by which plasmids could be screened for ssb expression. In addition, the F factor ssb gene (F ssb) was available on a fragment suitable for Southern hybridization experiments. Using both of these approaches the ssb gene carried by the IncI1 plasmid ColIb-P9 was identified. The IncP plasmid RP4 was shown to lack such an ssb sequence, in contrast to a previous report.

It was not convenient to use ColIb directly for the construction of a mutant defective in the ColIb ssb gene. Random mutations, such as transposon insertions, may disrupt controlling elements rather than the gene under investigation, and mutagenesis of specific plasmid sequences is difficult due to the size of the plasmid (93.2 kb). It was consequently necessary to construct recombinant plasmids carrying the ColIb ssb gene. A mutation could subsequently be introduced into the gene and transferred into the parental plasmid by recombination. Since it was therefore important that the mutation be easily selectable, transposon mutagenesis was initially attempted, using Tn723 which specifies kanamycin resistance (Ubben and Schmitt, 1986). However, a unique restriction site within the gene could also be used to introduce a selectable marker carried on a suitable restriction fragment and this
approach is also described. Since the mode of regulation of ColIb \textit{ssb}
may give clues to its likely function, particularly if the gene is
coordinately regulated with the transfer functions, this chapter also
describes investigations into the control of ColIb \textit{ssb} expression.

Specific fragments of ColIb-P9 are referred to throughout. These
are \textit{EcoRI} (E) or \textit{Sall} (S) digestion products and are numbered such that
1 is the largest fragment.

### 2.1 \textbf{ColIb}drd-1 specifies an \textit{ssb-1} suppressing activity.

To determine whether plasmid ColIb-P9 encoded an \textit{ssb-1} suppressing
activity plasmid pLG221 (Chatfield \textit{et al.}, 1982), a ColIb\textit{drd-1}
derivative containing Tn5 within the colicin Ib structural gene \textit{cib}),
was introduced by conjugation into an \textit{ssb-1} mutant strain of \textit{E. coli},
KL4330. The resulting strain was capable of growth at 44°C and showed
increased resistance to UV irradiation (Table 2.1.1; Fig. 2.1.1),
indicating that the plasmid suppressed \textit{ssb-1} detects. When a ColIb
plasmid naturally repressed for expression of transfer functions was
present in the \textit{ssb-1} strain, no suppression of the temperature
sensitivity phenotype was observed (See ColIb, Table 2.1.1).

Apparently, therefore, the gene responsible for \textit{ssb-1} suppressing
activity is coordinately controlled with the transfer functions. The
expression of this activity is discussed further in Section 2.5.

Suffice it to say at this point that the result suggested a role for
the putative ColIb \textit{ssb} gene in some aspect of conjugative DNA
processing.

As a first step towards constructing recombinant plasmids carrying
the ColIb gene causing \textit{ssb-1} suppressing activity, it was necessary to
determine the approximate location of the gene on ColIb. This
information could conveniently be obtained by screening an existing
cosmid library constructed using the pEMLcos4 vector and containing
TABLE 2.1.1 Suppression of the temperature sensitivity of KL4330 by plasmids

<table>
<thead>
<tr>
<th>Plasmid(s) present</th>
<th>KL4330 survival$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$6.8 \times 10^{-5}$</td>
</tr>
<tr>
<td>pLG221</td>
<td>0.74</td>
</tr>
<tr>
<td>Col1b</td>
<td>$2.4 \times 10^{-5}$</td>
</tr>
<tr>
<td>RP4</td>
<td>$9.9 \times 10^{-7}$</td>
</tr>
<tr>
<td>pKAC50</td>
<td>0.19</td>
</tr>
<tr>
<td>pBG373</td>
<td>$4.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>pCH11</td>
<td>0.30</td>
</tr>
<tr>
<td>pCH12</td>
<td>0.85</td>
</tr>
<tr>
<td>pACYC184</td>
<td>$2.9 \times 10^{-5}$</td>
</tr>
<tr>
<td>pCH14</td>
<td>0.96</td>
</tr>
<tr>
<td>pCH15</td>
<td>$1.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>pCH16</td>
<td>$1.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>pLG273</td>
<td>0.76</td>
</tr>
<tr>
<td>pLG273, pCH16</td>
<td>0.56</td>
</tr>
</tbody>
</table>

$^a$ Colony formation at 44°C relative to that at 30°C.
FIG. 2.1.1. Survival of ssb-1 strains after UV irradiation.

Strains were KL4330 (●) and derivatives harbouring pLG221 (○), pACYC184 (Δ), pCH4 (◼), pCH5 or pCH6 (■). Results, except for pACYC184, are the mean of at least 4 independent experiments.
fragments of approximately 45kb of Collbdrd-1 (Rees et al., 1987). After infection of the ssh-1 strain with this cosmid DNA, qualitative streak tests for growth at 44°C were used to identify suppressing cosmids. Such cosmids showed one of four distinct \textit{Eco}RI restriction patterns (Fig. 2.1.2), each including a common fragment corresponding to the largest \textit{Eco}RI fragment of Collbdrd-1 (E1, 20.3 kb). This fragment is overlapped by two \textit{Sal}I generated fragments, S3 (16.0 kb) and S4 (10.1 kb) and pBR328-based recombinants containing these fragments were already available (Rees et al., 1987).

The S4 recombinant was maintained stably only in a strain which also carried Collb. Attempts to isolate the S4 recombinant by transformation of standard laboratory strains (BW85, BW86) as well as KL4330, consistently resulted in the isolation of plasmids carrying deletions within the S4 fragment. In addition, after transformation of the \textit{rec}A mutant strain, BW103, the plasmid could not be reisolated. One derivative of the S4 recombinant, designated pCIII, was selected and shown by restriction analysis to have suffered a deletion of 2.35 kb within S4 (Fig. 2.1.3, Fig. 2.1.4). Nevertheless, this plasmid suppressed the temperature sensitivity of KL4330, implying that the S4 fragment specifies the ssh-1 suppressing activity, and that this is not affected by the deletion. The recombinant plasmid carrying S3 showed no such suppression. However, the S4 fragment is contained intact within another pBR328 derivative, pLG2001, which carries the whole E1 fragment of Collb-P9 (Merryweather et al., 1986; Fig. 2.1.4). This suggests that some function encoded within E1 is required for S4 stability, explaining the stabilizing effect of Collb on the S4 recombinant.

The 2.35 kb deletion suffered by the isolated S4 derivative was mapped using two \textit{Acc}I sites located within pCIII (Fig. 2.1.4). The intact S4 fragment was first isolated from a \textit{Sal}I digest of pLG2001 by electroelution from a 0.7% agarose gel and \textit{Acc}I digests of this
FIG. 2.1.2. Restriction maps of Colib-P9.

The innermost circle shows the Colib kilobase coordinates. The lines marked with arrowheads represent the extent of Colib DNA carried by four different cosmids which suppressed the ss6-1 temperature sensitivity phenotype of KL4330. The concentric rings marked with radial lines represent, from inner to outer, the fragments generated by EcoRI and Sall digestion of Colib, numbered in order of size within each map. Map data is from Uemura and Mizobuchi, 1982 and C.E.D. Rees, PhD. Thesis, University of Leicester, 1986.
2.1.3 Mapping of plasmid pCH1.

Digests and restriction fragments obtained (kilobases) were as follows:

<table>
<thead>
<tr>
<th>LANE</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
<th>g</th>
<th>h</th>
<th>i</th>
<th>j</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLASMID</td>
<td>pCH1</td>
<td>pLG2001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIGEST</td>
<td>PstI EcoRI EcoRI AvaI AvaI ClaI SalI SalI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PstI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRAGMENT SIZES</td>
<td>5.95</td>
<td>4.75</td>
<td>12.8</td>
<td>5.3</td>
<td>5.95</td>
<td>4.35</td>
<td>7.95</td>
<td>12.7</td>
</tr>
<tr>
<td>3.95</td>
<td>3.95</td>
<td>7.5</td>
<td>2.75</td>
<td>3.95</td>
<td>4.95</td>
<td>10.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.9</td>
<td>2.9</td>
<td>1.2</td>
<td>2.9</td>
<td>1.2</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Markers in lanes (a) and (b) are λcl857 Sam7 HindIII and HindIII-EcoRI digests respectively. The map constructed from this data is shown in Fig. 2.1.4. Note that in lane (j) the 7.95 kb fragment corresponds to the deleted 10.2 kb fragment of pLG2001 (lane j).
FIG. 2.1.4. Restriction maps of plasmids carrying ColIb ssβ

Line (a) represents kilobase coordinates on the ColIb map (Rees et al., 1987). Line (b) shows the intact E1 fragment, which is carried by plasmid pLG2001 inserted into the pBR328 EcoRI site. The location of ColIb ssβ is indicated. Plasmids shown below are linearized at one end of their vectors (thick lines; pBR328 for pCH1 and pCH2, pACYC184 for pCH4 and pCH6). Plasmid pCH5 (not shown) differs from pCH6 only in the orientation of the PstI insertion. Broken lines between lines (b) and (c) indicate the extent of the deletion present in pCH1, and between lines (e) and (f) the PstI insertion site. The location and direction of transcription of the KmR determinant of the insertion is also indicated. Restriction sites are AccI (A), ClaI (C), EcoRI (E), HindIII (H), PstI (P), and SalI (S).
fragment and of pCHI were then compared (Fig. 2.1.5). Since the fragment corresponding to the 2.7 kb Accl fragment of pLC2001 was absent from the pCHI digest it was concluded that, during the isolation of pCHI, the equivalent fragment of the S4 recombinant had suffered the deletion (see Fig. 2.1.4).

2.2 Collb-P9 carries a gene homologous to F ssb.

To determine whether the ssb-I suppression determined by Collb was due to the expression of a gene homologous to F ssb, Southern hybridization experiments were carried out using a fragment carrying the F ssb gene as a probe. Plasmid pKAC50 (Fig. 2.2.1) carries the F ssb gene on a 0.7 kb Avall fragment, and suppresses the temperature sensitivity of KL4330 at 44°C (Kolodkin et al., 1983; Table 2.1.1.) Southern hybridization of the 0.7 kb fragment of pKAC50 to the E1 and S4 fragments of Collbdrd-I digests confirmed that Collb carries an F ssb homologue, which was designated Collb ssb (Fig. 2.2.2). The F ssb probe also hybridized to a single 3.95 kb fragment from a ClaI-SalI double digestion of plasmid pCHI, locating Collb ssb to within this fragment (Fig. 2.2.2). Hybridization to two PstI fragments of 5.9 kb and 2.9 kb, and two ClaI-PstI fragments of 3.5 kb and 1.1 kb, also from digests of pCHI, indicated that the gene contained a PstI site, identified as that at coordinate 19.2 on Collb (Fig 2.2.2; Fig.2.1.4). The relative intensity of the bands obtained suggested that the 1.1 kb ClaI-PstI fragment, within the 3.95 kb ClaI-SalI fragment, contained the bulk of the gene (Fig. 2.1.4). Although this 3.95 ClaI-SalI fragment corresponds to a 6.3 kb fragment of Collb which had suffered a deletion during the isolation of pCHI, this deletion was known to have occurred approximately 2.5 kb from the PstI site thought to be present within Collb ssb (Fig. 2.1.4; see above). The size of the Collb ssb coding region was estimated from the known sequence of F ssb (Chase
FIG. 2.1.5. Mapping of the deletion in pCH1.

Lane (1) shows Accl digestion products of the intact S4 fragment of Collb, isolated from plasmid pLG2001. Fragment sizes are 4.6 kb, 2.8 kb and 2.7 kb. Lane (2) shows Accl digestion products of pCH1. Note that Accl also cuts at Sall restriction sites. The largest fragment in this lane (~4.9 kb) corresponds to the pBR328 vector. Two other bands are visible, one of ~4.6 kb and one of ~2.8 kb. The ~2.7 kb fragment of the S4 digest is missing from that of pCH1 and therefore must have suffered the deletion.
FIG. 2.2.1. Plasmid pKAC50.

The top line shows the F plasmid kilobase coordinates. The fragment shown is within the F3 fragment. The vector is a 2.3 kb portion of pBR322 running from the PvuII site at coordinate 2066 to the CiaI site at coordinate 23. The location and direction of transcription of F ssb are shown by the arrow. The 0.7 kb AvaiI fragment was that used in the Southern hybridization experiments described in the text. Map data is from Kolodkin et al., 1983 and Golub and Low, 1986.

Restriction sites are AvaiI (Av), BamHI (B), CiaI (C), PvuII (P) and Smal (S).
pKAC50

P

Ap^r

C

Av BP

Av

Av S/P

ssb
FIG. 2.2.2. Results of Southern hybridization of 0.7 kb fragment carrying F sex to restriction fragments of ColIb, RP4 and pCH4:

A. Agarose gel electrophoresis of RP4 cut with PstI (1), StuI (2), and PstI and StuI (3) and of ColIbdrd-1 cut with SalI (5) and EcoRI (6). λcl857 cut with EcoRI and HindIII is shown as marker (4).

B. Southern blot analysis of (A).
FIG. 2.2.2. Results of Southern hybridization of 0.7 kb fragment carrying F $ssb$ to restriction fragments of ColIb, RP4 and pCH4:

C. Digests of pCH1 cut with ClaI and PstI (1), ClaI and SalI (2) and PstI (3).

D. Corresponding Southern analysis. Molecular sizes (kb) are indicated.

E. Hybridization, using total pKAC50 Avall digest as probe, to 2 fragments of ~28.2 kb and ~2.6 kb from the PstI digest of RP4 and one fragment of ~14.8 kb from the SstII digest. These fragments correspond to those carrying the bla determinant of RP4 (see Fig. 2.3.1.).
et al., 1983a) to be approximately 0.55 kb and it was therefore unlikely that the deletion was closer than about 2.0 kb to the gene. Thus the *Clal-Sall* fragment of *pCH1* carrying the *Collb ssb* gene was deemed suitable for the construction of a *Collb ssb* mutation.

In order to obtain a recombinant plasmid carrying *Collb ssb* on a smaller fragment of *Collb*, the 3.95 kb *Clal-Sall* fragment identified as carrying the *Collb ssb* gene was inserted into the *Tc* determinant of *pBR328* by ligation of *Clal-Sall* digested *pCH1* and vector DNA. This DNA was used to transform strain KL4330, with selection for the *pBR328* specified chloramphenicol resistance. Colonies were then selected for growth at 44°C and for tetracycline sensitivity. Plasmid DNA was prepared and analysed by restriction digestion (Table 2.2.1). One representative plasmid, *pCH2* (Fig. 2.1.4), was shown to efficiently suppress the temperature sensitivity of the *ssb-1* strain (Table 2.1.1).

2.3 The broad-host-range plasmid RP4 lacks an *F ssb* homologue.

Since plasmid RP4 had been reported to carry an *F ssb* homologue, this plasmid was also investigated in parallel studies to those already described. However, RP4 did not suppress the temperature sensitivity of KL4330 (Table 2.1.1). In addition, the *F ssb* probe did not hybridize to a digest of the plasmid in Southern hybridizations, even when stringency was reduced by use of a 3x SSC rather than 1x SSC final wash (Fig. 2.2.2). However, *Avall* digested total pKAC50, when used as probe, apparently hybridized to the ampicillin resistance determinant (*bla*) of RP4, as detailed in figures 2.2.2. and 2.3.1. The size of the hybridizing fragments in this experiment corresponded closely to those previously reported to carry an *F ssb* homologue (Golub and Low, 1985), suggesting that the result obtained by Golub and Low was due to contamination of the probe with vector sequences. It was therefore concluded that RP4 did not carry a gene homologous to *F ssb*, and no
### TABLE 2.2.1. Mapping of plasmid pCH2.

Digests and restriction fragments obtained were as follows:

<table>
<thead>
<tr>
<th>PLASMID: pCH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIGEST:</td>
</tr>
<tr>
<td>PstI EcoRI EcoRI Aval Aval Clal Clal SalI PstI SalI</td>
</tr>
<tr>
<td>FRAGMENT SIZES:</td>
</tr>
<tr>
<td>4.95 4.95 8.4 4.4 3.65 4.45 8.4 8.4 3.45 2.3 4.0 3.1 3.95 1.2 0.4</td>
</tr>
</tbody>
</table>

The map constructed from this data is shown in Fig. 2.1.4.
FIG. 2.3.1. Restriction map of RP4

Numbers indicate kilobase coordinates. The Ap°, Tc° and Km°
determinants are indicated. Lines outside the circle represent
fragments which hybridized to the total pKAC50 AvaII digest.
Restriction sites are EcoRI (E), PstI (P) and SstII (S). Data is from
Thomas and Smith (1987)
further work was carried out with this plasmid.

2.4 Construction of an insertional mutation of Collb ssb.

Once the Collb ssb gene had been obtained on a recombinant plasmid it was possible to introduce a mutation into the gene. Initially, attempts were made to introduce random insertions into the cloned Collb ssb gene using transposon Tn1723, which specifies kanamycin resistance (Ubben and Schmitt, 1986). The pCH2 derivative used for this procedure was pCH3, a plasmid which differs from pCH2 only by the removal of the unique EcoRI site within the vector by fill-in synthesis and blunt-end ligation. The rationale for the construction of this plasmid was that the bulk of any Tn1723 insertion within pCH3 could be excised at the EcoRI sites located near each end of the transposon. A 35 base-pair insert would then remain and could be detected using colony hybridization techniques. Strain RU2537, which carries the transposon within the bacterial chromosome, was transformed with pCH3 DNA. Following incubation at 30°C, the optimum temperature for transposase activity (Kretschmer and Cohen, 1979), plasmid DNA was prepared from Ap' colonies. A transposon-free strain was transformed with this DNA with selection for Ap'Km' transformants. Unfortunately, Ap'Km' cells obtained using this method were found to contain pCH3 derivatives which were in the form of multimers, as judged by restriction analysis (Fig. 2.4.1). Disruption of one Collb ssb gene could therefore not be detected as other intact copies of the gene were present on each plasmid.

In an attempt to overcome the problem of multimerization, the 3.95 kb ClaI-SalI fragment of pCH2 was inserted into the tetracycline resistance determinant of plasmid pACYC184 (Chang and Cohen, 1978). In comparison to the pBR328-based recombinants, the resulting plasmid, pCH4 (Fig. 2.1.4), showed significantly reduced levels of multimerization. Plasmid pCH4 was constructed by first cutting pCH2
FIG. 2.4.1 Multimerization of plasmids carrying Tn1723 insertions.

The parental plasmid used was pCH3, a derivative of pCH2 differing only by the removal of the unique EcoRI restriction site. Lanes 2 to 7 are EcoRI digests of six pCH3::Tn1723 isolates. Tn1723 itself contains a single EcoRI site and multimers are therefore apparent in these digests, as plasmid copies which do not carry the transposon remain uncut. Lanes 8 and 9 show EcoRI digests of pCH2 and pCH3 respectively. Lanes 10 to 17 are EcoRI - PstI double digests of the same pCH3::Tn1723 plasmids, while lanes 18 to 21 show uncut plasmid DNA of two of the Tn1723 containing isolates, pCH2 and pCH3 respectively. The last two lanes are λc1857 Δsam7 HindIII and HindIII - EcoRI markers.
with Clal, SalI, and EcoRI restriction endonucleases to excise the Clal-SalI fragment required and to cut within the vector, reducing the probability of reisolating the plasmid. This DNA was then ligated with Clal-SalI digested pACYC184 and used to transform KL4330, with selection for chloramphenicol resistance. Colonies obtained were tested by replica plating for ampicillin or tetracycline sensitivity. Ampicillin sensitivity would indicate the absence of pBR328, while tetracycline sensitivity should result from insertion of the Clal-SalI fragment into pACYC184. Finally, Ap⁰Tc⁰ colonies were tested for growth at 44°C before analysis of plasmid DNA by EcoRI-PstI digestion (Table 2.4.1). To ensure that growth of such strains was not due to an ssb reversion, a fresh isolate of KL4330, known to be temperature sensitive, was transformed with the plasmid and colonies tested for ssb-I suppression (Table 2.1.1; Fig. 2.1.1). Suppression of both ssb-I phenotypes by pCH4 demonstrated that the cloned fragment alone is sufficient for ssb-I suppression.

Southern hybridization studies had already determined that a PstI site was present within the region of Colle homologous to F ssb. This allowed an alternative approach to the introduction of a mutation into Colle ssb, as the insertion of a suitable restriction fragment within this site would probably disrupt Colle ssb expression. In addition, the insertion could carry a selectable marker. This approach would involve ligating plasmid DNA linearized at the PstI site with a PstI fragment carrying an antibiotic resistance determinant. Thus the problems associated with multimerization should be avoided since, as the recombinant plasmid would be linearized, even if multimers were to occur they should only form after the introduction of the insertion. Plasmid pUC4.K (Vieira and Messing, 1982) contains a suitable PstI fragment carrying a kanamycin resistance determinant specifying aminoglycoside 3'-phosphotransferase (APH). This is carried within a
TABLE 2.4.1. Mapping of plasmid pCH4.

Digests and restriction fragments obtained (kilobases) were as follows:

<table>
<thead>
<tr>
<th>PLASMID:</th>
<th>pCH4</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIGEST:</td>
<td>SalI</td>
</tr>
<tr>
<td>FRAGMENT SIZES:</td>
<td>7.35</td>
</tr>
<tr>
<td></td>
<td>3.40</td>
</tr>
</tbody>
</table>

The map constructed from this data is shown in Fig. 2.1.4.
HaeII fragment derived from Tn903 (Oka et al. 1981). The kanamycin resistance gene was excised on an approximately 1.2 kb PstI fragment from pUC4.K and ligated into the unique PstI site of pCH4 (see Fig. 2.1.4). Kanamycin resistant KL4330 transformants were tested for loss of ssb-1 suppressing activity, and plasmids from temperature sensitive strains were screened by EcoRI-PstI and ClaI-SalI digestion (Table 2.4.2). A ClaI site within the Km' insertion allowed a pair of plasmids to be identified which carried the insertion in opposite orientations (pCH5 and pCH6, see Fig. 2.1.4). Neither of these plasmids suppressed the temperature or UV sensitivity phenotypes of the ssb-1 strain, and in fact appeared to sensitize the cells to UV irradiation (Table 2.1.1; Fig. 2.1.1). Thus, expression of ColIb ssb had been disrupted by the Tn903-derived insertion and the PstI site probably lies within the gene. Plasmid pCH6 did not affect ssb-1 suppression at 44°C by pLG273, a ColIb'd-1 derivative, indicating that the ColIb ssb mutation was recessive (Table 2.1.1).

2.5 ColIb ssb is coordinately expressed with the ColIb transfer genes.

Preliminary investigations had already indicated that the ColIb ssb gene was expressed coordinately with the genes required for conjugative DNA transfer. Since this suggested that ColIb ssb may have a role in conjugation, it was important for the interpretation of ColIb SSB function to confirm this observation. As outlined in Section 2.1, only a ColIb derivative derepressed for expression of transfer functions was able to suppress the temperature sensitivity of an ssb-1 mutant of E. coli. This is confirmed by data (Table 2.5.1) showing that pLG273 confers a significantly higher level of suppression than the repressed ColIb plasmid, pLG272. Two other plasmids, pLG274.2 and pLG276.3, which carry transposon insertions which prevent the positive regulation of the transfer functions, were both unable to suppress the
TABLE 2.4.2. Mapping of plasmids pCH5 and pCH6.

Digests and restriction fragments obtained (kilobases) were as follows:

<table>
<thead>
<tr>
<th>PLASMID:</th>
<th>pCH5</th>
<th>pCH6</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIGEST:</td>
<td>EcoRI</td>
<td>CiaI</td>
</tr>
<tr>
<td></td>
<td>PstI</td>
<td>SalI</td>
</tr>
<tr>
<td>FRAGMENT SIZES:</td>
<td>4.65</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>2.7</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>1.4</td>
</tr>
</tbody>
</table>

The map constructed from this data is shown in Fig. 2.1.4.
### TABLE 2.5.1 Suppression of the temperature sensitivity of KL4330 by plasmids

<table>
<thead>
<tr>
<th>Plasmid(s) present</th>
<th>KL4330 survival $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$6.8 \times 10^{-5}$</td>
</tr>
<tr>
<td>pLG273</td>
<td>0.76</td>
</tr>
<tr>
<td>pCH4</td>
<td>0.96</td>
</tr>
<tr>
<td>pLG272</td>
<td>$1.4 \times 10^{-5}$</td>
</tr>
<tr>
<td>pLG274.2</td>
<td>$3.8 \times 10^{-5}$</td>
</tr>
<tr>
<td>pLG276.3</td>
<td>$7.2 \times 10^{-5}$</td>
</tr>
<tr>
<td>pLG272, pCH4</td>
<td>0.65</td>
</tr>
<tr>
<td>pLG274.2, pCH4</td>
<td>$4.3 \times 10^{-2}$</td>
</tr>
<tr>
<td>pLG276.3, pCH4</td>
<td>0.52</td>
</tr>
<tr>
<td>pLG339</td>
<td>$2.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>pCH14</td>
<td>0.11</td>
</tr>
</tbody>
</table>

$^a$ Colony formation at 44°C relative to that at 30°C.
Mutation at 44°C (Table 2.5.1). This supports the conclusion that Colb 

ssb is under coordinate control with the transfer genes. Plasmid pCH4 was still able to suppress ssb-I at 44°C in strains also harbouring pLG272, pLG274.2 or pLG276.3, confirming that the suppression phenomenon requires the plasmid SSB and not some undefined tra gene product.

The ability of Colb and Colbdrd-I derivatives to suppress the ssb-I UV sensitivity at 30°C was also investigated. In marked contrast to the temperature sensitivity data, the results shown in Fig. 2.5.1 indicate that derepression of the transfer functions is not a prerequisite for suppression of the UV sensitive phenotype conferred by the E. coli ssb-I mutation.

It was therefore apparent that although plasmid SSB was required for suppression of both the temperature and UV sensitivity phenotypes, only suppression of the former required derepression of transfer gene expression. These apparently contradictory results could be reconciled if the ssb gene of Colb were expressed at two levels. A basal low level expression might be sufficient for suppression of UV sensitivity, whereas suppression of temperature sensitivity might require a higher level of expression, obtained when the Colb transfer system is derepressed. The ability of pCH4 to suppress the ssb-I mutation at 44°C in the absence of the positive regulators required for high level expression may reflect the high copy number of the pACYC184 vector, approximately 20 per chromosome (Chang and Cohen, 1978). Expression from the fragment cloned in pCH4 possibly results in high cellular concentrations of the Colb ssb product, sufficient for suppression of ssb-I temperature sensitivity and accounting for the apparent discrepancy.

This possibility was tested by inserting the 3.95 kb Clal-Sall fragment of pCH4 carrying Colb ssb into the tetracycline resistance
FIG. 2.5.1 Survival of strains after UV irradiation.

Strains were KL4330 (▲) and derivatives harbouring pLG272 (■), pLG273 (□), pLG274.2 (○), pLG276.3 (●) or pCH14 (△). Results are the mean of at least 2 independent experiments.
determinant of pLG339, a pSC101 derivative with a copy number of six to eight per chromosome (Stoker et al., 1982). The resulting plasmid (pCH14, Fig.2.5.2) suppressed the temperature sensitivity of \(ssb^{-1}\) cells less efficiently than pCH4, supporting this hypothesis (Table 2.5.1). Both low and high copy number recombinant plasmids conferred similar levels of UV resistance on the \(ssb^{-1}\) strain (Fig. 2.5.1).

In conclusion, it appears that the Collb \(ssb\) gene is naturally expressed at a low basal level but with a higher level of expression being achieved when the genes required for conjugal transfer are derepressed. Although lying outside the defined transfer regions of Collb (Rees et al., 1987) the Collb \(ssb\) gene therefore probably encodes a product involved in some aspect of the conjugal transfer process.

2.6 Introduction of the Collb \(ssb\) mutation into Collb\(drd^{-1}\).

The next step towards an analysis of the role of the Collb \(ssb\) product was to introduce the mutation carried by pCH6 into Collb\(drd^{-1}\). Since Collb \(ssb\) was thought to have some involvement in conjugal transfer a Collb derivative carrying a Collb \(ssb\) mutation might be expected to show some deficiency in this process. The aim of further investigations would therefore be to search for such deficiencies. This would be best achieved if the parental plasmid were derepressed \(drd\) for expression of the transfer functions.

The protocol followed for the transfer of the Collb \(ssb\) mutation into an appropriate Collb derivative was that of Winans et al., (1985). 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 a \(recBC\ sbcB\) strain carrying the target replicon itself. This strain lacks exonuclease \(V\) activity, preventing degradation of the linearized plasmid (Wachernagel, 1973), while the
FIG. 2.5.2. Plasmid pCH14.

The vector portion (thick line) is derived from pLG339 (Stoker et al., 1982). The 3.95 kb Clal-Sal fragment carrying ColIb ssb derived from pCH4 (thin line) is inserted within the Tc\(^r\) determinant of the vector. The position and direction of transcription of ColIb ssb and of the Km\(^r\) specified by the vector is indicated (arrows). Radial lines indicate kilobase coordinates. Restriction sites are Clal (C), HindIII (H), PstI (P) and SalI (S).
*sbcB* mutation, which inactivates exonuclease I, restores the recombinational activity eliminated by the *recBC* mutation [Kushner et al., 1972]. The linear molecule can only be maintained via integration into another replicon by a double recombination event and this should predominantly occur between the sequences flanking the mutation and the homologous sequences at the target site.

Following this scheme, JC7623, the *recBC sbcB* strain, was transformed initially with plasmid pLG273. Plasmid pCH6 was linearized by digestion with *EcoRI* and *SalI*, the double digest reducing the possibility of recircularization. This DNA was then used to transform JC7623 (pLG273) with selection for the kanamycin resistance specified by the insertion. Colonies obtained were screened for chloramphenicol sensitivity, which would indicate the absence of the recombinant plasmid, and plasmid DNA was prepared. An increase, from 10.1 kb to approximately 11.6 kb, in the size of the S4 fragment in plasmids from Km" colonies obtained from this procedure, compared to that of pLG273, indicated that the insertion had occurred at the correct location. Eight such isolates were introduced into KL4330. Two plasmids, pCH8 and pCH9 obtained from independent experiments, failed, in contrast to pLG273, to suppress either the UV or temperature sensitivity phenotypes caused by the bacterial *ssb-I* mutation [Table 2.6.1; Fig. 2.6.1]. These plasmids probably carried the Collb *ssb* mutation transferred from pCH6. The restoration of *ssb-I* suppressing activity when pCH4 was introduced by transformation into the KL4330 (pCH8) strain [Table 2.6.1; Fig. 2.6.1] indicated that the defect was due to disruption of the Collb *ssb* gene in pCH8 and confirmed that the mutation was recessive.

Confirmation that the Km" *PstI* cassette had been introduced into the S4 fragment was obtained by Southern hybridization with the 1.2 kb Km" fragment isolated from pUC4.K. Hybridization occurred to both the E1 and S4 fragments of pCH8, and to a 1.2 kb fragment from a *PstI*
## TABLE 2.6.1 Suppression of the temperature sensitivity of KL4330 by plasmids

<table>
<thead>
<tr>
<th>Plasmid(s) present</th>
<th>KL4330 survival $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$6.8 \times 10^{-5}$</td>
</tr>
<tr>
<td>pLG273</td>
<td>0.76</td>
</tr>
<tr>
<td>pCH4</td>
<td>0.96</td>
</tr>
<tr>
<td>pCH8</td>
<td>$1.1 \times 10^{-5}$</td>
</tr>
<tr>
<td>pCH9</td>
<td>$3.4 \times 10^{-6}$</td>
</tr>
<tr>
<td>pCH8,pCH4</td>
<td>0.29</td>
</tr>
</tbody>
</table>

$^a$ Colony formation at 44°C relative to that at 30°C.
FIG. 2.6.1. Survival of ssb-1 strains after UV irradiation.

Strains were KL4330 (●) and derivatives harbouring pCH4 (△), pLG273 (□), pCH8 or pCH9 (■) or both pCH4 and pCH8 (○). Results were the mean of at least 4 independent experiments.
digest of this plasmid (Fig. 2.6.2). No hybridization was apparent with
digests of pLG273.

This information, together with loss of ssh-1 suppressing activity
by the mutant plasmid, indicates that the insertion disrupts the Collb
ssh gene of pCH8. In addition, the 2.35 kb deletion present in pCH6 has
not been introduced into pCH8, as a deletion of this size would be
apparent from the restriction analysis data.

2.7 Discussion.

The results presented in this chapter have shown that, in common
with many other conjugative plasmids, the InclI plasmid Collbdrd-I
suppresses ssh-1 defects due to the expression of a gene homologous to
F ssh. It had previously been reported that plasmid RP4 contained a
sequence which hybridized to the F ssh gene carried on a 0.7 kb AvaII
fragment of plasmid pKAC50 (Golub and Low, 1985). However, in contrast
to this report, results obtained here, using a probe identical to that
used by Golub and Low, indicated that RP4 did not carry an F ssh
homologue. The implication of this finding is discussed in Chapter
Seven. The fragments of RP4 previously claimed to hybridize to the F
ssh probe correspond closely in size to the PstI fragments of RP4
carrying the Apr determinant. Thus the result obtained by Golub and Low
was probably due to contamination of the probe with sequences from the
pBR322-derived vector component of pKAC50, since this also carries an
Apr determinant.

A recombinant plasmid carrying the S4 fragment of Collb, which
contains the Collb ssh gene, was not maintained stably in the absence
of Collb. However, a stable derivative (pCH1) was obtained which had
suffered a 2.35 kb deletion. The instability of the S4 recombinant may
be due to an activity encoded within S4 which is regulated by some
other Collb product, and in the absence of this regulation becomes
FIG. 2.6.2. Results of Southern hybridization of -1.2 kb PstI fragment from pUC4.K to digests of pCHB and pLG273.

A. Agarose gel electrophoresis of pCHB cut with EcoRI (a), SalI (b) and PstI (c) of pLG273 cut with EcoRI (d) and of pUC4.K cut with PstI (e).

B. Southern blot analysis. Molecular sizes (kb) are indicated.
lethal to the cell. Plasmid pCH1 may have been stabilized following a recombinational event which resulted in deletion of the portion of S4 responsible. The inability to isolate the S4 recombinant after transformation of the recA mutant strain, in which such an event is inhibited, supports this hypothesis. The nature of the system responsible for S4 instability has not been elucidated but could be similar to the \( \text{kil-kor} \) activity of many IncP plasmids (Figurski et al., 1984). This similarly prevents isolation of specific fragments from these plasmids due to expression of genes (\( \text{kil} \)) which are lethal to the host in the absence of \( \text{trans} \)-acting controlling elements (\( \text{kor} \) or \( \text{kil} \)-override). \( \text{kil} \) and \( \text{kor} \) genes are possibly part of a complex system involved in replication and plasmid maintenance (Shingler and Thomas, 1984). Of course, such a functional interpretation cannot yet be applied to the results described here.

The deletion within pCH1 was shown to have occurred approximately 2.0 kb from ColIb \( \text{ssb} \) and consequently did not impede further progress. A recombinant plasmid could subsequently be constructed which carried the ColIb \( \text{ssb} \) gene on a 3.95 kb fragment derived from pCH1. Initial attempts to introduce a mutation within the ColIb \( \text{ssb} \) gene carried by derivatives of this plasmid were not successful due to the multimerization of the recombinant. The multimerization of these pBR328-derived plasmids may be due to the absence of a \( \text{cer} \) site, a \( \text{cis} \) acting recombination site required for the efficient resolution of ColE1 plasmids (Summers and Sherratt, 1984). However, a \( \text{PstI} \) site located within ColIb \( \text{ssb} \) could be utilized for the construction of a ColIb \( \text{ssb} \) mutation by inserting into this site a fragment encoding the \( \text{Kmr} \) determinant of Tn903. This eliminated the \( \text{ssb-1} \) suppressing activity of the recombinant and had therefore disrupted ColIb \( \text{ssb} \). This mutation was successfully transferred into a ColIb\( \text{adr-1} \) derivative by recombination. The \( \text{ssb} \) gene of ColIb had therefore been mapped and a
mutation constructed suitable for further investigations into the role of the plasmid-encoded single-stranded DNA binding protein.

Preliminary results had suggested that ColIb ssb may be regulated coordinately with the genes for conjugative transfer, indicating that the ColIb ssb product was involved in conjugation. Consequently, the regulation of ColIb ssb expression was also investigated. Although the ability to suppress the ssb-1 temperature sensitivity phenotype was found to be dependent on derepression of the ColIb transfer functions, all ColIb plasmids tested which carried an intact ColIb ssb gene suppressed the UV sensitivity of these strains, regardless of the level of transfer gene expression. It has therefore been postulated that ColIb ssb is expressed at two levels, a low basal level and a higher level coordinately expressed with the transfer functions. Possibly the ColIb ssb product is consitutively required for vegetative replication, but is required at higher levels for conjugative DNA processing.

Intuitively, and by analogy with the known role of E. coli SSB in DNA replication, it could be predicted that ColIb SSB may play a role in complementary strand synthesis in the recipient or replacement strand synthesis in the donor, or both, during conjugative transfer.
Chapter Three

The nucleotide sequence of Collb ssb

Introduction.

The aim of the work described in this chapter was to determine the nucleotide sequence of the Collb ssb gene. This was carried out principally to ascertain whether the Tn903-derived insertion within pCH6 actually disrupted the Collb ssb coding sequence and whether any resulting truncated protein was likely to retain biological activity. Knowledge of the Collb ssb sequence would also allow its comparison with the known sequences of other ssb genes, particularly those of E. coli and the F sex-factor (Sancar et al., 1981; Chase et al., 1983). Analysis of the predicted amino acid sequence would allow the prediction of the secondary structure of the protein which could also be compared with similar analyses of the F and E. coli proteins. Some indication of the relatedness of these genes could then be obtained, possibly allowing the functional domains of the three proteins to be elucidated further.

Until recently DNA sequencing techniques have generally relied on the use of M13 cloning vectors in order to obtain the single-stranded DNA required for the sequencing reaction (Messing et al., 1977). These techniques require extensive subcloning of fragments into M13 and the use of time consuming methods for DNA preparation. However, plasmid sequencing techniques are now available that rely on denaturation of double-stranded plasmid DNA, prepared using simple small-scale methods, to provide the single-stranded template. This can be used in conjunction with the chain terminator sequencing method (Sanger et al., 1977). A plasmid sequencing technique was therefore used for the work described here since it would allow the existing recombinants carrying Collb ssb to be utilized directly.

As a first step towards the sequencing of a cloned gene it is
necessary to construct primers complementary to a known sequence lying close to the gene. Such primers must prime extension from a unique sequence within the plasmid to prevent initiation from other undetermined sites. This sequence should also be devoid of any extensive secondary structure which might otherwise impede hybridization with the primer. Primers should ideally allow sufficient data to be obtained to minimize the number of additional primers required to complete the sequence determination.

Plasmid pCH4 carries ColIb ssb on a 3.95 kb fragment and no suitable restriction sites were found for the excision and subcloning of a smaller fragment carrying the gene. The extent of DNA flanking ColIb ssb would therefore preclude direct sequence determination using a primer corresponding to a known vector sequence. However, the sequence of the insertion within ColIb ssb, carried by pCH6, was already known, since it was derived from a Haell fragment of Tn903 (Oka et al., 1981; Vieira and Messing, 1982). It would therefore be possible to use primers corresponding to sequences within this insertion to obtain the initial data required for the complete determination of the ColIb ssb gene sequence.

This chapter describes the determination of the nucleotide sequence of ColIb ssb using this approach and the analysis of the data obtained.

3.1 Determination of the nucleotide sequence of ColIb ssb.

As plasmid pCH6 carried an insertion of known sequence within the ColIb ssb gene, primers corresponding to sequences at either end of the insertion would allow a large portion of the gene sequence to be determined. From the original published sequence of Tn903 (Oka et al., 1981), it was apparent that inverted repeats were present at each end of the insertion in pCH6. Consequently it would not be possible to use
primers corresponding to part of this repeated sequence in conjunction with intact pCH6 template DNA, since any sequencing reaction would then progress simultaneously in both directions from the insertion. However, the extent of the terminal repeats (approximately 230 bp) made unique sequences further within the insertion unsuitable as primer sites. This was because sequence data can only be clearly read up to about 300 bp from the primer and such primers would therefore only allow approximately 70 bp of sequence to be determined on either side of the insertion.

In order to allow more extensive sequence data to be obtained it would be necessary to isolate fragments of pCH6 carrying only one of the Tn903-derived repeat sequences. This could be achieved by cutting pCH6 at the HindIII site located within the insertion and at the EcoRI site within the vector. Two restriction fragments of approximately 5.0 kb and 3.0 kb result from this procedure and these can be conveniently isolated by electroelution from a 0.7% agarose gel. Although these fragments could be ligated into suitable cloning vectors, there was apparently no obstacle to using the purified fragments directly as template DNA in separate sequencing reactions.

Before this could be attempted a suitable primer was first obtained which corresponded to a sequence near one end of the Tn903-derived insertion. This primer (5' AGCAACACCTTCTTCACG) corresponds to a sequence present 16 bp from each end of the HaeII fragment used to construct pUC4.K (primer 1; see Fig. 3.1.1) and was used in sequencing reactions in which the two EcoRI-HindIII fragments of pCH6 acted as templates. However, following autoradiography of the resulting sequencing gels it was not possible to determine any significant portion of the ColIb ssb sequence. The failure to obtain satisfactory results using these fragments is probably due to the difficulty in obtaining adequate concentrations of DNA, as each fragment had to be
FIG. 3.1.1. Nucleotide and predicted amino acid sequence of the ColIb ssb gene and protein.

The amino acid sequence of the F and E. coli SSBs (Chase et al., 1983; Sancar et al., 1981) are shown only where differences from ColIb occur. Gaps introduced to maximize homology are indicated by asterisks. The PstI site into which the Tn903-derived Km" determinant was inserted is shown underlined, as is the AccI site which is present in all three nucleotide sequences. Sequences corresponding to the primers used for the sequencing reactions, and the direction of extension from these primers, are represented by arrows numbered 1 to 5. Primer 1 corresponds to a sequence located within the insertion, 27 bp downstream of the PstI site. Primers 4 and 5 correspond to sequences located 48 bp upstream and 57 bp downstream of the ColIb ssb coding sequence, respectively. Sequence numbers correspond to the residues of the ColIb protein. The orientation of ColIb ssb is such that the direction of transcription is from right to left on the map shown in Fig. 2.1.4.
individually isolated from an agarose gel. Nevertheless, although the bands obtained were not clear, sufficient information was present to indicate that the primer was only initiating synthesis on the 3.0 kb EcoRI-HindIII fragment of pCH6. In reactions in which the 5.0 kb EcoRI-HindIII fragment was used as template results were apparently identical to those obtained using the 3.0 kb template, probably due to the presence of small concentrations of the 3.0 kb fragment. This indicated that initiation of synthesis was only occurring on the smaller of the two fragments, suggesting that a deletion may have occurred at one end of the insertion in pCH6. Consequently it was possible to use the same primer in reactions using intact pCH6. This was preferable to using the isolated fragments since much higher concentrations of DNA could easily be prepared. In this way the sequence of approximately 230 bp was obtained from one strand of Collb ssh to the left of the inserted fragment as shown in Fig. 2.1.4.

The data obtained using this method was sufficient to allow two further primers to be made, corresponding to the sequences 5' GAGGCGCACTCCGTACC and 5' AAGAATTTCACTGATGTA located within the 230 bp region of Collb ssh already sequenced (see primer 2 and primer 3 in Fig. 3.1.1.). These primers initiate extension in opposite directions such that the two sequence readings obtained overlap. Plasmid pCH4 was used as template, as this recombinant carries the intact Collb ssh gene. The two primers were then used to obtain the sequence extending from the primer sites to 70 bp upstream of the ATG translation initiation codon and to 80 bp downstream of the TCA termination codon (Fig. 3.1.1; Fig. 3.1.2).

The complete sequence of Collb ssh could be determined from the data obtained in the reactions described above. However, the sequence most distant from the primer sites could not be read sufficiently clearly to exclude the possibility that errors were present in these
FIG. 3.1.2 Comparison of sequences upstream (A) and downstream (B) of the coding regions of Col1b ssβ, F ssβ and E. coli ssβ.

The presumptive Shine-Dalgarno, ATG initiation codons and TGA termination codons are underlined. The orientation of Col1b ssβ is such that the direction of transcription is from right to left on the map shown in Fig. 2.1.4. The data for the F and E. coli sequences are from Chase et al., 1983 and Sancar et al., 1981, respectively. Arrows indicate the primers used for sequencing reactions, and the asterisks indicate the two mismatches between primer number four and its priming site.
FIG. 3.1.2

A.  
Collb  ssb  CTTTCGCGTCCTGCTGAAGGGACACATGAACGTCCCGTCTCGGGTATGCGGTTTAT
F    ssb  GTTCCCCGCAAAGGTGAAACCCGGCTGCCGAATCTCAAAGATGAGATGGGCT
E. coli ssb  ACAACATAGTAAAAAGCGCTATTGGTAATGGTACAACACCGCGTTTACACTTATCA

CTCACATCTTTTCAGTCAAGAGGTCTTATG
GAAAACATCAACAGAAGGAGACACATCATG
GAACGATTTTTTTCAGGAGACACCAACATG

B.  
Collb  ssb  TGATCGGGCTACTGCTGGAACCCCGCCCGTCTGTGCGGGCATCACCGGAGATAA
F    ssb  TGAACGACTGACTGTGACACCGCCCCGTCCTGTGCGGGGCACCCGGAGAGATG
E. coli ssb  TGATTTGTCAATAAAACATAGTTATATTGTTTTAAGGTGATGATTAAAGCATC

TGAGGATGAGCGAATATTTCAGACCTGCCCGGACCGCTGCCTT
AGGATGAGCGAATATTTCAGAATACCTCAG
TGCCAGCCATAAAAGAAGCCTCGTTAT
regions of the deduced sequence. In addition, runs of consecutive identical nucleotides are often difficult to resolve as discrete bands on the sequencing gel and other ambiguities also often arise. However, such problems can usually be overcome by sequencing the complementary strand of the gene.

Only the sequence of 168 bp in the region sequenced using both primer 1 and primer 3 had already been determined on both strands of Collb ssb. Primers were therefore subsequently made which corresponded to the sequences 5' GGCCACATTGAACCGTCC and 5' CTGAAATATTCGCTCATC (see primer 4 and primer 5, Fig. 3.1.1; Fig 3.1.2) which had previously been determined and were thought to lie 48 bp upstream and 57 bp downstream of the gene, respectively. These primers should then have allowed the complete nucleotide sequence of Collb ssb to be obtained from the complementary strands of pCH4 to those already sequenced. Primer 4 would also allow the 5' end of the insertion in pCH6 to be sequenced, and thus determine whether a deletion was responsible for the failure of primer extension described above.

Primer 5 allowed the sequence determination of 340 bp at the 3' end of the gene. Unfortunately, primer 4 did not initiate elongation. Consequently the sequence of 93 bp downstream of the initiation codon was only determined on the one strand sequenced using primer 3. However, reactions using primer 3 and the pCH4 template were repeated, and a gel run for 5 hours, rather than the usual 1.5 or 3 hours. This allowed much greater resolution of the data corresponding to sequences upstream of Collb ssb and to the 100 bp region at the 5' end of the gene. It was then apparent that primer 4 contained two mismatches with the primer site on pCH4, which accounted for its inability to initiate synthesis. Nevertheless, the sequencing of the 5' region of the gene had been repeated and the resolution of the gel was such that it is unlikely that errors are present in the deduced Collb ssb sequence.
Tracks are (1) plasmid pCH4 sequenced using primer 2 (see Fig. 3.1.1) and (2) plasmid pCH4 sequenced using primer 3 (Fig. 3.1.1). Sequencing was by the chain terminator method (Sanger et al., 1977). The tracks labelled A, C, G, and T refer to reactions terminated with ddATP, ddCTP, ddGTP and ddTTP, respectively.
The sequence of the 528 nucleotides which constitute the Collb ssb gene is presented in Fig. 3.1.1. along with the predicted amino acid sequence of the SSB protein. Since the F and E. coli SSBs both lack the initiating methionine it is assumed, in view of the sequence similarities, that the same is true of the Collb protein. The predicted number of residues in Collb SSB is therefore 174 and the protein has a calculated molecular weight of 19,110. In Fig. 3.1.2. the sequences upstream and downstream of Collb ssb are compared with the presumptive F plasmid ssb and E. coli ssb promoter and terminator sequences described previously (Sancar et al., 1981; Chase et al., 1983).

This work was carried out in collaboration with Dr. Barth of ICI Pharmaceuticals Division who has, using the primers described above, both confirmed the sequence of the Collb ssb gene and extended the sequence information to 142 bp upstream and 132 bp downstream of the gene.

3.2 Analysis of the nucleotide sequence of Collb ssb

In order to assess the similarities between the Collb protein and those of F and E. coli, computer analyses were carried out on the predicted amino acid sequences of these proteins. Hydropathy profiles were generated based on the values of Kyte and Doolittle (1982) and the plots generated by this program are compared in Fig. 3.2.1. These hydropathy profiles are virtually identical for the three proteins. Since the secondary structure of a protein depends to a large extent on the distribution of hydrophilic and hydrophobic residues this result indicates that the three proteins are likely to have very similar secondary structures.

The comparison of protein secondary structure can be pursued further by Chou and Fasman analysis which allows the prediction of regions of α-helix and β-pleated sheet structure by a method which uses
FIG. 3.2.1 Hydropathy profiles of ColIb SSB (A), F SSB (B) and *E. coli* SSB (C) calculated using the values of Kyte and Doolittle (1982) and plotted against amino acid sequence number.
FIG. 3.2.2 Prediction of secondary structure of Collb SSB (A), F SSB (B) and E. coli SSB (C) using the method of Chou and Fasman (1978). Numbers refer to the amino acid sequence. Secondary structure is represented as follows: Alpha-helix (\[\backslash / \backslash\]); Beta-sheet (\[\backslash / \backslash / \backslash / \backslash\]); Turn (\[\_\_\_\_\_\]); Random coil (\[\_\_\_\_\_\_\_\_\_\_\_\]). Circles represent hydrophobic regions.
data based on the statistical analysis of proteins with known secondary structure (Chou and Fasman, 1978). The diagrams shown in Fig. 3.2.2. compare the Chou and Fasman predictions for the three SSB proteins. Although differences are apparent, regions containing extensive secondary structure are often associated with functional domains, and the distribution of such regions is very similar in each of the three proteins. This suggests the existence of conserved functional domains between these proteins.

3.3 Discussion.

From the sequencing data presented in this chapter it is apparent that the Collb ssb gene shares considerable homology with both the F-plasmid and E. coli ssb genes. There is 84% sequence homology between the two plasmid genes at the nucleotide level, and 83% at the predicted amino acid level. Collb SSB lacks four residues present in F SSB, but these lie in the region (residues 130 to 140) that is highly variable between all three SSBs. The Collb protein also shows 60% sequence identity with the E. coli protein and these two proteins share many extensive regional homologies. Chou and Fasman computer analysis of the amino acid sequences of the three proteins suggests that the secondary structure of each is similar. The three proteins are therefore probably both functionally and evolutionarily related.

A further homology has been detected between the Collb SSB sequence and that of the polypeptide encoded by open reading frame one of the trfA (trans-acting replication function) operon of the IncP plasmid RK2 (Smith and Thomas, 1984; Thomas and Smith, 1987; Fig. 3.3.1). This polypeptide consists of 116 amino acids and may be responsible for the Kild phenotype associated with the trfA operon, although this has not been proven (Thomas and Smith, 1987). The Kild phenotype inhibits plasmid maintenance when not controlled by a kor (kil-override) gene, designated trfB. The 28% homology between Collb
SSB and this trfA polypeptide is predominantly in the region corresponding to the predicted DNA-binding domain of Collb SSB.

It is interesting that although a Shine-Dalgarno sequence is present upstream of the Collb ssb gene, no recognisable promoter or transcriptional terminator is apparent in the deduced sequence. In addition, the Collb ssb sequence is flanked by open reading frames which read in the same direction as it, suggesting that Collb ssb may be part of an operon.

A primer which initiated synthesis from within one terminal repeat of the Km\(^r\) insertion in pCH6 initially allowed a portion of the Collb ssb sequence downstream of the insertion to be determined. Thus, it is apparent that this insertion must disrupt the Collb ssb gene itself. It was not possible to sequence the 5' end of Collb ssb using the same primer, suggesting that there was a mismatch between the primer and one of the terminal repeat sequences. It was thought that this was probably due to a deletion at one end of the insertion, although it was not clear at which stage this deletion had occurred. However, the lack of primer extension in one direction is explained by the recent report that, during the construction of pUC4.K, a deletion was sustained near one end of the Tn903-derived fragment (Taylor and Rose, 1988). This deletion is of 220 bp and, except for 6 bp, has entirely removed the inverted repeat at the 5' end of the kanamycin resistance gene. The orientation of the insertion within pCH6 was already known (see Chapter Two). Thus it was possible to determine that the deletion was present towards the 5' end of Collb ssb. This is in agreement with the position of the deletion which would be predicted from the results described above.

The PstI site in pCH4, into which the Km\(^r\) fragment was inserted, is located 94 bp from the ATG initiation codon of Collb ssb. Due to the failure of the primer corresponding to a sequence upstream of Collb ssb
FIG. 3.3.1. Comparison of the RK2 trfA ORF 1 polypeptide and the predicted amino acid sequence of ColIb SSB. The amino acid sequence of the 116 amino acids encoded by open reading frame 1 of the trfA operon of plasmid RK2 [Smith and Thomas, 1984; Thomas and Smith, 1987] is shown only where differences from ColIb SSB occur. Gaps introduced to maximize homology are indicated by asterisks, and conservative amino acid substitutions are shown underlined.
Collb ssh Ser Ala Arg Gly Ile Asn Lys Val Ile Leu Val Gly Arg Leu Gly
RK2 trfA * * Met Ser His Gln Phe Gln Phe Ile Asn Thr

Asn Asp Pro Glu Val Arg Tyr Ile Pro Asn Gly Gly Ala Val Ala Asn Leu Glu
Arg Thr His Gly Asn Ser Asn Lys Pro Gln Ile Phe Asp

Val Ala Thr Ser Glu Ser Trp Arg Asp Lys Gln Thr Gly Glu Met Arg Glu Gln
Ile Val Asn Glu Asn * Asp Ala Asp Lys Glu Arg

Thr Glu Trp His Arg Val Val Leu Phe Gly Lys Leu Ala Glu Val Ala Gly Glu
Asp Phe Phe Ile Lys Cys Ser Glu Ala Gin Lys

Tyr Leu Arg Lys Gly Ala Gin Val Tyr Ile Glu Gly Gln Leu Arg Thr Arg Ser
Gly Ser Leu Phe Val Gin Lys Ile Asn Thr Lys

Trp Asp Asp Asn Gly Ile Thr Arg Tyr Ile Thr Glu Ile Leu Val Lys Thr Thr
Tyr Glu Lys Asp Gln Val Gly Asp Phe Ile Ala Asp Lys Val

Gly Thr Met Gln Met Leu Gly Ser Ala Pro Gln Gln Asn Ala Gin Ala Gin Pro
Asp Tyr Leu Asp Thr Lys Ala Pro Gly Gly Ser Asn Gin Glu END

Lys Pro Glu Gln Asn Gly Gin Pro Gln Ser Ala Asp Thr Lys Lys Gly Gly Ala

Lys Thr Lys Gly Arg Gly Arg Lys Ala Ala Gin Pro Glu Pro Glu Pro Glu Thr

Pro Glu Gly Glu Asp Tyr Gly Phe Ser Asp Ile Pro Phe
(Primer 4; see Fig. 3.1.1) to initiate synthesis, it has not been possible to confirm the location of the deletion within the insertion. However, from the sequence of Collb ssb and the information published by Taylor and Rose (1988) it is possible to predict the size of the truncated protein which is expressed as a result of the Collb ssb mutation. A TAA termination codon lies 61 bp within the pUC4.K-derived fragment at the 5' end of the Kmr determinant. It can therefore be predicted that the Collb ssb truncated polypeptide is a protein of approximately 5.8 kDa consisting of 53 or 52 amino acids, depending on whether the initiating methionine residue is present or absent. The predicted amino acid sequence of this truncate is shown in Fig 3.3.2.

Since only 31 amino acids from the Collb ssb sequence are present in this truncated protein it is unlikely that the protein would retain its original biological activity. The single-stranded DNA-binding site within both the \( \xi \) coli and F SSB proteins is thought to be located within the region between residues 29 and 115, which also includes the site of the \( ssb-I \) mutation of the \( \xi \) coli protein (Chase et al., 1983; Prasad and Chiu, 1987). The \( PstI \) insertion site in Collb ssb, which seems to be similarly located to the \( PstI \) site within F ssb, is present towards the amino-terminal end of this region. The truncated protein has therefore probably lost the ssDNA binding site and hence the associated binding activity. It can therefore be concluded that the insertion within Collb ssb carried by pCH6 and pCH8 disrupts the coding region of the gene and results in the expression of a truncated protein which is unlikely to retain a DNA binding activity. It should be noted that the direction of transcription of the kanamycin resistance determinant within the insertion is the same as that of Collb ssb. Since no strong transcription terminators are present downstream of the Kmr determinant (Oka et al., 1981), readthrough may occur into sequences downstream of the insertion.
FIG. 3.3.2 Nucleotide and amino acid sequence of predicted truncated polypeptide encoded by ColIb ss6 mutant.

The PsI insertion site and the predicted termination codon within the insertion are underlined. The position of the 220 bp deletion within the insertion is also marked (Ψ). A portion of the nucleotide sequence of the kanamycin resistance gene specified by the insertion is shown in italics. Sequence data for the insertion is from Oka et al. (1981) and Taylor and Rose (1988).
FIG. 3.3.2

ATG ACT GCA CGT GGT ATC AAC AAG GTC ATC CTC GTC GGG CGT CTG GGC AAT GAT
Met Ser Ala Arg Gly Ile Asn Lys Val Ile Leu Val Gly Arg Leu Gly Asn Asp
PstI

CCG GAG GTC CTG TAC ATC CCC AAC GGT GGC GCA GTG GCA AAC CTG CAG GGG GGG
Pro Glu Val Arg Tyr Ile Pro Asn Gly Gly Ala Val Ala Asn Leu Gln Gly Gly

GGG GGA AAG CCA CGT TGT GTC TCA AAA TCT CTG ATG TTA CAT TGC ACA AGA TAA
Gly Gly Lys Pro Arg Cys Val Ser Lys Ser Leu Met Leu His Cys Thr Arg End

AAA TAT ATC ATG AAC AAT AAA ACT GTC TGA TTA CAT AAA CAG TAA TAC AAG
End

GGG TGT TAT GAG CCA TAT TCA ACG GGA AAC GTC TGC GAG GCC GCG ATT AAA

TTC CAA CAT GGA TGC TGA TAT ATA TGG GTA TAA ATG GCC TCG CGA TAA TGT CCG
End

End

End
Chapter Four

Collb ssh is in the leader region.

Introduction

The work presented in this chapter describes the determination of the direction of conjugative transfer of Collb-P9. The ssh gene of Collb maps close to the origin of transfer (oriT) and it was of interest to determine whether this gene was in the leader region of the plasmid, the region transferred early during conjugation, as is the case for the ssh gene of plasmid F. It was also of interest to compare the organization of the Collb transfer genes with that of other conjugative plasmids for which the direction of transfer and tra gene organization was already known.

The low-level transfer of chromosomal genes from donor cells harbouring an integrated IncI1 plasmid, R483, already indicates that transfer of plasmids specifying the I1 conjugation system is unidirectional (Datta and Barth, 1976). However, the direction of transfer has not been reported. The oriT site of Collb had previously been shown by mobilization tests using cloned fragments of the plasmid to be located on a 1.55 kb PstI fragment within the E1 fragment of the plasmid (Rees et al., 1987). A recombinant plasmid (pLG2008) carrying this fragment was available. Furthermore, this PstI fragment contains single ClaI and BglII restriction sites which allow its orientation to be determined within both Collb and the recombinant plasmid.

Boyd and Sherratt (1986) had recently determined the direction of transfer of the small mobilizable plasmid ColE1. By inserting the cloned ColE1 oriT site into a λ dv vector it was possible to integrate the oriT site specifically within a λ prophage by homologous recombination between vector and prophage sequences. Thus the oriT site was integrated at a specific site, and in known orientation, within the bacterial chromosome. The necessary ColE1 mobilization genes (mob) were
provided on a recombinant plasmid and the bacterial genes were
mobilized from the integrated oriT by a conjugative plasmid (R64dred-
II). The frequency of mobilization of bacterial genes allowed the
direction of transfer from the integrated oriT site to be determined. A
similar technique to that used by Boyd and Sherratt was used in this
work to determine the direction of transfer of ColIb-P9.

4.1 Construction of λdx plasmids carrying the ColIb oriT.

The method used by Boyd and Sherratt (1986) to introduce the oriT
site of ColEI into the bacterial chromosome requires that the cloned
fragment carrying oriT be first inserted into a λdx vector. λdx
plasmids are fragments of phage λ carrying the cro, o and p genes and
which are consequently able to replicate autonomously. Plasmids pCB104
and pCB105 (Fig. 4.1.1) are λdx vectors carrying a lacZ'-polylinker
region on a Haell fragment derived from pUC9 (Boyd and Sherratt, 1986;
Vieira and Messing, 1982) and a Haell-Hhal fragment of pACYC184
encoding the chloramphenicol resistance gene (Alton and Vapnek, 1979;
Boyd and Sherratt, 1986; Chang and Cohen, 1978). These plasmids cannot
replicate in a cell producing active cI repressor since expression of
cro is turned off under these conditions, preventing replication
(Matsubara, 1981). However, at a low frequency these plasmids can
survive in a λ lysogen by integrating into the bacterial chromosome by
homologous recombination. Integration should occur preferentially at
the region of the prophage homologous to the λ sequences carried by the
plasmid. Thus a cloned fragment carried by a λdx vector can be
specifically integrated at a known site within the bacterial chromosome
in known orientation.

The first step towards constructing a mobilizable chromosome
containing the ColIb origin of transfer was therefore to insert the
cloned PstI fragment carrying the oriT site into the lacZ'-polylinker
FIG. 4.1.1. Plasmid pLG253.

The vector is pCB104 (4.3 kb), which is related to the λ dλ vectors described by Boyd and Sherratt (1986). The insert (heavy line) is the 1.55 kb PsI fragment containing Collb oriT (see Fig. 4.1.2; Fig. 4.1.3). BgIII (B), CiaI (C), EcoRI (E), HindIII (H), and PsI (P) sites are indicated. Also shown are the λ genes, the lacZ′-polylinker region, and the chloramphenicol resistance (Cm') determinant. The lower part of the figure represents integration of the plasmid into λWJB360 prophage by homologous recombination. The numbers at the bottom show the map positions of gal, biot, and trp on the E. coli K-12 linkage map. The arrow indicates the inferred direction of transfer from the integrated oriT fragment with gal in the leading region. Vector pCB105 is identical to pCB104 except that the small HaeII (Δ) fragment containing the polylinker is reversed.
region of the \( \lambda dv \) vector pCB104. The 1.55 kb \( PstI \) fragment of ColIb containing the \( oriT \) was isolated from a \( PstI \) digest of the recombinant plasmid pLG2008 (Fig. 4.1.2), and ligated with \( PstI \) digested pCB104 DNA. Recombinants were selected by loss of \( \alpha \)-complementation. Plasmid DNA was prepared from colonies carrying the recombinant and the orientation of the cloned fragment in one recombinant, designated pLG253, was determined by restriction analysis (Fig. 4.1.2; Fig. 4.1.3). A complementary plasmid containing the \( PstI \) \( oriT \) fragment in the opposite orientation was also constructed. This was achieved by ligating the small \( EcoRI-HindIII \) fragment from pLG253 into the polylinker of pCB105, which is identical to pCB104 except that the \( HaeII \) fragment carrying \( LacZ^+ \) and the polylinker is reversed (Fig. 4.1.1). The orientation of the insertion in the resulting plasmid, pLG254, was confirmed by restriction analysis (Fig. 4.1.4).

4.2 Insertion of the ColIb \( oriT \) site into the \( E. coli \) chromosome.

The next step towards introducing the \( PstI \) \( oriT \) fragments carried by plasmids pLG253 and pLG254 into the chromosome involved recombining these fragments into the \( \lambda \) insertion vector WJB360. The region of homology between the plasmids and this \( \lambda \) vector spans approximately 2.5 kb. Strain CB123, a \( \lambda WJB360 \) lysogen (Boyd and Sherratt, 1986), was transformed independently with pLG253 and pLG254, with selection for the \( Cm^+ \) phenotype specified by the recombinants. RecA-dependent integration of the \( \lambda dv \) vectors had been reported by Boyd and Sherratt (1986) to be inefficient, with less than 10 \( Cm^+ \) colonies formed per \( \mu g \) plasmid DNA. The transformation of CB123 was therefore carried out using a DNA concentration of approximately 2\( \mu g \) per 100\( \mu l \) (3\( \times 10^8 \)) bacterial cells. A yield of 5-6 \( Cm^+ \) colonies per \( \mu g \) DNA was obtained using this procedure.

The WJB360 prophage is temperature-inducible due to the presence
FIG. 4.1.2. Map of Collb-P9 showing the exclusion (exc), single-stranded-DNA-binding protein (ssb) and DNA primase (sog) genes. Radial lines indicate EcoRI restriction sites. The expansion to the right shows the orientation of the PstI fragment (1.55 kb) containing the oriT site, which is located in the ~0.9 kb CiaI-TaqI fragment. The arrow indicates the inferred direction of transfer of Collb with ssb in the leading region. BglII (B), CiaI (C), PstI (P), and TaqI (T) restriction sites are shown. Map data is from Rees et al. (1987), see Chapter One.
FIG. 4.1.3. Mapping of plasmid plG253.

Digests and restriction fragment sizes obtained were as follows.

<table>
<thead>
<tr>
<th>LANE</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIGEST:</td>
<td>EcoRI</td>
<td>HindIII</td>
<td>EcoRI</td>
<td>HindIII</td>
</tr>
<tr>
<td>Calfi</td>
<td>Calfi</td>
<td>BglII</td>
<td>BglII</td>
<td></td>
</tr>
<tr>
<td>FRAGMENT SIZES:</td>
<td>5 3</td>
<td>4.71</td>
<td>2.97</td>
<td>3.60</td>
</tr>
<tr>
<td></td>
<td>0.48</td>
<td>1.07</td>
<td>1.42</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>0.68</td>
<td>0.65</td>
<td>0.65</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Markers in lanes 1 and 6 are \( \Phi X174 \) HaeIII and \( \lambda c1857 \) Sam7 HindIII-EcoRI digests, respectively.
FIG. 4.1.4. Mapping of plasmid pLG254.

Digests and restriction fragment sizes obtained were as follows:

<table>
<thead>
<tr>
<th>LANE</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIGEST:</td>
<td>EcoRI</td>
<td>EcoRI</td>
<td>HindIII</td>
<td>HindIII</td>
</tr>
<tr>
<td>Clal</td>
<td>BglII</td>
<td>Clal</td>
<td>BglII</td>
<td></td>
</tr>
<tr>
<td>FRAGMENT SIZES:</td>
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<td>3.84</td>
<td>4.71</td>
<td>2.97</td>
</tr>
<tr>
<td></td>
<td>0.48</td>
<td>0.67</td>
<td>1.07</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>0.55</td>
<td>0.87</td>
<td>0.55</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Markers are φX174 HaeIII digests (lanes 1 and 10), λcl857 Sam7 HindIII digests (lanes 2 and 9) and λcl857 Sam7 HindIII-EcoRI digests (lanes 3 and 8).
of a class allele (Boyd and Sherratt, 1986). Transducing phages carrying the integrated oriT fragment in opposite orientations were thermally induced at 40°C from the two Cm CB123 transformant strains. Lysates produced in this way were used to make Cm lysogens of strain C600. Small-scale plasmid-preparation techniques were then used to demonstrate that these strains did not contain autonomously replicating plasmids and that the Cm phenotype must therefore be conferred by integrated sequences. Strains carrying insertions derived from pLG253 and pLG254 were designated BW105 and BW107, respectively. C600 lysogens carrying λWJB360 Δ pCB104 or λWJB360 Δ pCB105 prophage were also constructed in the same way to serve as oriT-negative control strains. These strains were designated BW106 and BW108, respectively.

Confirmation that the four strains described above carried integrated λΔv-derived sequences within a λ prophage was obtained by assessing the ability of thermally induced lysates to transduce C600 to chloramphenicol resistance.

4.3 Mobilization of bacterial genes from the integrated ColIb oriT.

The necessary functions required for the mobilization of the chromosome from the integrated ColIb oriT were provided by introducing, by conjugation, the ColIbΔrd-I plasmid pLG273 into each of the four test strains, with selection for the tetracycline resistance specified by this plasmid. The resulting strains were then used as donors in filter matings with a streptomycin resistant λ lysogen, BW39(λ+). This strain carries mutations in gal (min 17), pro (min 7), arg (min 77), his (min 44) and trp (min 28). Mating was carried out for 90 min and incubations were at 30°C. The yield of Tc transconjugants was routinely approximately 10⁸ per mating. The frequency of mobilization by pLG273 of extrachromosomal copies of pLG253 and pLG254 was also determined by mating C600 strains harbouring both the λΔv plasmid and
pLG273 with BW39. The two λdv-oriT plasmids showed cotransfer frequencies of 0.38 and 0.25, respectively, in matings carried out under identical conditions to those described above.

The results shown in Table 4.3.1 indicate that there was a low frequency of transfer of chromosomal markers from the BW106 and BW108 control strains, which lack an integrated oriT site. However, there was little or no bias in the relative yields of the Gal^ and Trp^ recombinants in these matings. The higher frequencies of recombinants detected when the donor cells carried an oriT prophage indicate chromosomal mobilization. Values for chromosome mobilization from BW105 donors indicate that gal^ was transferred about 70-fold more frequently than trp^ from the integrated oriT site. The gradient of transfer of the five chromosomal markers selected (Fig. 4.3.1), implies a preferred direction of transfer, shown by the arrow in Fig. 4.1.2. The results obtained from mobilization of the BW107 chromosome (Table 4.3.1; Fig. 4.3.1) demonstrate that inversion of the oriT site reversed the bias in the relative yields of chromosomal markers, implying that the direction of transfer from the Collb oriT site is unidirectional. Since the orientation of the oriT fragment in both Collb and the λdv vectors was known, the direction of transfer of Collb can be deduced to be anticlockwise according to the map in Fig. 4.1.2.
TABLE 4.3.1. Mobilization of chromosomal genes from the ColIb
oriT site inserted in a λ prophage.

<table>
<thead>
<tr>
<th>Donor strain and ADV plasmid</th>
<th>Recombinants per pLG273 transconjugant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Str&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Gal&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>BW105 (pLG253)</td>
<td>1.2x10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>BW106 (pCB104)</td>
<td>2.9x10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>BW107 (pLG254)</td>
<td>3.2x10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>BW108 (pCB104)</td>
<td>2.6x10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are the mean of three independent experiments.
nt; not tested
FIG. 4.3.1. Mobilization of chromosomal markers from integrated Colib origin of transfer. Markers were gal (min 17), pro (min 7), arg (min 77), his (min 44) and trp (min 28). Recombinants obtained per pLG273 transconjugant are plotted against the relative distance of each marker from the oriT site.
4.4 Discussion.

The results presented in this chapter have indicated that the direction of transfer of ColII is unidirectional, such that the ColII ssb gene is transferred early while the tra genes are transferred last. The unidirectional transfer of ColII found in this work is in agreement with the observed polar transfer of chromosomal genes from cells carrying the integrated IncII plasmid R483 (Datta and Barth, 1976). The low background level of recombinants detected with the BW106 and BW108 donors, which did not carry the integrated oriT site, may reflect integrative fusion of the IncII plasmid and the bacterial chromosome (Boyd and Sherratt, 1986).

Chromosomal mobilization obtained using the system described in this chapter was relatively inefficient compared to the mobilization of the extrachromosomal copies of the λdv-oriT plasmids. However, a similar deficiency was found with chromosome mobilization from the integrated ColEI origin of transfer, mediated by another IncII plasmid, R64ard-11. This deficiency was attributed to inefficient initiation and rapid termination of DNA transfer (Boyd and Sherratt, 1986).

The relative sequence of gene transfer found with ColII is known to be the same for three other, apparently unrelated, conjugative plasmids. These plasmids, F (IncFI; Ippen-Ihler and Minkley, 1986), RP4 (IncP; Al-Doori et al., 1982; Grinter, 1981) and R46 (IncN; Coupland et al., 1987) are thought to be quite distinct, but in each case the oriT site is located close to one end of the tra region, as in ColII (Ippen-Ihler and Minkley, 1986; Thomas and Smith, 1987; Winans and Walker, 1985). The naturally mobilizable plasmid ColEI also shows a similar arrangement in that transfer is unidirectional with the genes required for mobilization [mob] transferred last (Boyd and Sherratt, 1986). Thus the transferable plasmids studied so far are all organised such that the transfer genes are transmitted last to the recipient cell.
Since these plasmids do not appear to be closely related, this arrangement may have some functional significance. This may relate to a requirement in the recipient cell for the late transfer of the \textit{tra} genes. Neither complementary strand synthesis on the transferred strand nor circularization of the plasmid require the expression of plasmid genes in the recipient (Hiraga and Saitoh, 1975; Boulnois and Wilkins, 1978). Possibly the transfer of the \textit{tra} region or the expression of the \textit{tra} region or the expression of the transfer genes in the recipient cell acts as a signal for the termination of conjugation, triggering the active disaggregation of the mating cells, the final stage in the conjugative process (Willetts and Skurray, 1980). Alternatively the arrangement described may relate to a requirement for prolonged expression of the \textit{tra} genes in the donor cell, or may be a consequence of a requirement for the early transfer of other genes, such as those involved in DNA synthesis, to the recipient cell. These points are discussed further in Chapter Seven.

It is apparent that the \textit{ssb} genes encoded by ColIb-P9, F, and presumably those plasmids showing homology to the leading region of F, are transferred early during conjugation. This might indicate a requirement for early expression of the \textit{ssb} gene following DNA transfer. However, this seems unlikely since the ColIb \textit{ssb} has been shown to be expressed at high level only in the presence of the two positive regulators of the ColIb \textit{tra} genes (see Chapter Two). Since the genes encoding these regulators are transferred late during conjugation, high level expression of \textit{ssb} would not be possible until after the completion of the transfer process. Thus a role for ColIb SSB in the recipient during conjugation seems unlikely unless the protein is transferred as a complex with the transferring single-strand. Expression of ColIb \textit{ssb} would then be required only in the donor cell, and there would be no necessity for the gene to be transferred early. This argument would also apply to any other early transferred genes,
which are coordinately regulated with the transfer functions. The possibility that Collb SSB is transported to the recipient cell bound to the transferred strand has been investigated with negative results (see Chapter Five; C. E. D. Rees, personal communication, 1988). A role for SSB in the donor cell is therefore favoured.

The functional significance, if any, of the clustering of homologous sequences, including the plasmid ssh, within the leading region of these plasmids remains unclear at present. However, the homology observed between the leading regions of these plasmids may reflect some ancestral relationship, as discussed in Chapter Seven.
Chapter Five

Properties of an ssh mutant of Collb\textsuperscript{\textmd{-I}}: Effect of the mutation on conjugative transfer and plasmid maintenance stability.

Introduction

The aim of the work described in this chapter was to determine whether any mutant phenotypes were associated with the Collb ssh mutation carried by the Collb\textsuperscript{\textmd{-I}} derivative pCH8, and by further investigation of any such phenotypes to determine the role of the Collb SSB protein. Results are presented in Chapter Two which indicated that Collb ssh is controlled coordinately with the conjugation genes. This finding suggested that the plasmid ssh genes may express products which have a role in conjugative DNA metabolism.

It was therefore predicted that the Collb\textsuperscript{\textmd{-I}} derivative carrying the Collb ssh mutation would show some defect in conjugation, detectable as a deficiency in the formation of transconjugant cells following mating. However, if Collb SSB were to be involved only in replacement strand synthesis in the donor cell, any defect in transconjugant formation might be minimal. Nevertheless, a defect in replacement strand synthesis might be expected to result in some loss of the plasmid from donor cells, since IncII plasmids have a low copy number of only one or two per bacterial chromosome (Jacob et al., 1977). This chapter therefore initially describes experiments designed to investigate both of these possibilities. A remaining possibility, suggested by the requirement for \textit{E. coli} SSB in the replication of single-stranded DNA phages, was that the plasmid SSB proteins may be involved in the vegetative replication of plasmid DNA. This was investigated by comparing the maintenance stability of the Collb ssh mutant and the parental plasmid.
5.1 Effect of ColIb ssb mutation on conjugation

If the ColIb SSB protein has a major role in the conjugative process, it would be expected that disruption of the ColIb ssb gene would result in a reduction in conjugative efficiency. The conjugative ability of the ColIb ssb mutant plasmid, pCH8, was therefore assessed by comparison with the parental ColIbdrd-I plasmid, pLG273.

It is possible that the E. coli SSB protein can compensate for the loss of the plasmid-encoded SSB and these experiments therefore initially used ssb-I mutant strains of E. coli. Matings were carried out at 44°C in order to inactivate the SSB-1 protein. However, at this temperature defects in bacterial metabolic processes due to the ssb-I mutation would be significant because ssb-I is a quick-stop mutation which results in cessation of DNA synthesis (Glassberg et al., 1979; Meyer et al., 1979). Plasmid pLG273 is able to suppress such defects, due to the expression of ColIb ssb, as described in Chapter Two. Since pCH8 is unable to suppress ssb-I defects, in matings at 44°C this mutant plasmid might show a deficiency in transconjugant formation not reflecting a direct involvement of the plasmid SSB in conjugation.

If the plasmid SSB protein plays a major role in plasmid DNA metabolism it may have an activity for which the bacterial SSB protein cannot substitute. Thus there would be a requirement for the plasmid protein despite the presence of an active bacterial SSB. If this were the case the ColIb ssb mutant plasmid might show defects in conjugation at temperatures which do not inactivate the SSB-1 protein.

It can also be hypothesized that any defect in bacterial conjugation resulting from the ColIb ssb mutation would be more easily detected under conditions which are optimal for the conjugative transfer process. Such conditions are met at 37°C. It was therefore necessary to allow for the possible effect of different temperatures on conjugation between ssb-I cells. Matings between ssb-I donors and
recipients were therefore carried out at a variety of temperatures ranging from 30°C to 44°C.

KL4330 donor strains were mated with KL4330R, a rifampicin resistant derivative of KL4330, and transconjugants selected on medium containing rifampicin and tetracycline. A donor to recipient ratio of 1:9 was used, as in all the matings described in this section. Thus there would be no deficiency of recipient cells in a 30 min mating.

Table 5.1.1 shows that the yield of transconjugants was highest when mating was at 37°C and that, at all temperatures tested, pCH8 formed approximately as many transconjugants in a 30 min mating as the parental plasmid, pLG273. This result indicated that the Collb ssb product was not essential for efficient transfer or the subsequent establishment of the plasmid in the transconjugant cells.

Although no defect in conjugation was detectable after a 30 min mating it was possible that any defect might become apparent after a longer mating period. Consequently the experiment was repeated under the same conditions but with the exception that mating was carried out for 60 min. In this case pCH8 was found to form approximately two-fold fewer transconjugants than pLG273 when mating was at 37°C or higher (Table 5.1.2). Thus, although there was no major defect in the conjugative process as a result of the Collb ssb mutation, the plasmid SSB may act to promote some aspect of conjugative DNA metabolism.

To further investigate this possibility, experiments were carried out in which samples were assayed at fifteen minute intervals up to 60 min. However, no large defect in pCH8 transfer was apparent at either 30°C (Table 5.1.3) or 44°C (Table 5.1.4) in these experiments.

Similarly, the efficiency of transconjugant formation was approximately the same for pCH8 and pLG273 in 30 min matings at 37°C between KL4330R donors and BW97, a standard ssb+ laboratory strain (Table 5.1.5). In matings between ssb+ donors (BW85) and recipients (BW97), a small
TABLE 5.1.1 Transconjunct formation in 30 min matings of KL4330 donors and KL4330R recipients at different temperatures.

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Transconjunctants formed with donors harbouring plasmid:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pLC273</td>
</tr>
<tr>
<td></td>
<td>/ml</td>
</tr>
<tr>
<td>30</td>
<td>1.5x10^7</td>
</tr>
<tr>
<td>37</td>
<td>3.8x10^7</td>
</tr>
<tr>
<td>40</td>
<td>1.8x10^7</td>
</tr>
<tr>
<td>42</td>
<td>2.0x10^7</td>
</tr>
<tr>
<td>44</td>
<td>1.5x10^7</td>
</tr>
</tbody>
</table>

Results are the mean of two independent experiments.
TABLE 5.1.2. Transconjugant formation in 60 min matings of KL4330 donors and KL4330R recipients at different temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Transconjugants formed with donors harbouring plasmid:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pLG273</td>
</tr>
<tr>
<td></td>
<td>/ml</td>
</tr>
<tr>
<td>30</td>
<td>9.3x10⁶</td>
</tr>
<tr>
<td>37</td>
<td>6.6x10⁷</td>
</tr>
<tr>
<td>40</td>
<td>2.3x10⁸</td>
</tr>
<tr>
<td>42</td>
<td>3.3x10⁸</td>
</tr>
<tr>
<td>44</td>
<td>6.9x10⁷</td>
</tr>
</tbody>
</table>

Results are the mean of two independent experiments.
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Transconjugants formed with donors harbouring plasmid:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pLG273</td>
</tr>
<tr>
<td></td>
<td>/ml</td>
</tr>
<tr>
<td>0</td>
<td>2.4x10^4</td>
</tr>
<tr>
<td>15</td>
<td>5.8x10^5</td>
</tr>
<tr>
<td>30</td>
<td>9.0x10^5</td>
</tr>
<tr>
<td>45</td>
<td>3.1x10^6</td>
</tr>
<tr>
<td>60</td>
<td>1.5x10^7</td>
</tr>
</tbody>
</table>

TABLE 5.1.3. Transconjugant formation in interrupted matings of KL4330 donors and KL4330R recipients at 30°C.
TABLE 5.1.4. Transconjugant formation in interrupted matings of KL4330 donors and KL4330R recipients at 44°C.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Transconjugants formed with donors harbouring plasmid:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pLG273</td>
</tr>
<tr>
<td></td>
<td>/ml</td>
</tr>
<tr>
<td>0</td>
<td>$7.0 \times 10^3$</td>
</tr>
<tr>
<td>15</td>
<td>$1.1 \times 10^5$</td>
</tr>
<tr>
<td>30</td>
<td>$5.0 \times 10^5$</td>
</tr>
<tr>
<td>45</td>
<td>$2.1 \times 10^6$</td>
</tr>
<tr>
<td>60</td>
<td>$4.4 \times 10^6$</td>
</tr>
</tbody>
</table>
TABLE 5.1.5. Transconjugants formed in 30 min matings at 37°C

<table>
<thead>
<tr>
<th>Donor Strain</th>
<th>Recipient Strain</th>
<th>Transconjugants formed/ml</th>
<th>Transconjugants formed/donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>KL4330R (pLC273)</td>
<td>BW97</td>
<td>$2.0 \times 10^7$</td>
<td>0.94</td>
</tr>
<tr>
<td>KL4330R (pCH8)</td>
<td>BW97</td>
<td>$2.5 \times 10^7$</td>
<td>0.99</td>
</tr>
<tr>
<td>BW85 (pLC273)</td>
<td>BW97</td>
<td>$5.9 \times 10^7$</td>
<td>2.31</td>
</tr>
<tr>
<td>BW85 (pCH8)</td>
<td>BW97</td>
<td>$4.0 \times 10^7$</td>
<td>1.75</td>
</tr>
</tbody>
</table>

Results with KL4330R donors are the mean of four independent experiments, those with BW85 donors are the mean of two independent experiments.
reduction in the transfer efficiency of pCH8 was detected (Table 5.1.5).

These results indicate that the ColIb ssb gene has no major role in either the transfer of plasmid DNA or its processing in the recipient cell, and also suggest that the E. coli SSB is not required for DNA transfer itself. However, the possibility remains that the inactivated SSB-1 protein is able to participate in some aspect of the transfer process. In addition, the SSB-1 protein is able to renature when returned to low temperature (Meyer et al., 1979) and, as cells were plated at 30°C after mating, the KL4330R SSB-1 protein would be active during the subsequent incubation period. Thus, these results do not rule out the possibility that the E. coli SSB functions in the conjugative processing of the transferred DNA strand in the recipient cell.

However, a defect in replacement strand synthesis in the donor cell might have only a minimal effect on transconjugant formation over a short mating period in which pre-existing DNA will be transferred to the recipient cell. Such a defect would probably not be detected in the experiments described above. The possibility therefore still remained that ColIb ssb may be involved in the synthesis of a replacement strand in the donor cell during transfer. As the copy number of ColIb-P9 is low (Jacob et al., 1977) it would be expected that, if ColIb SSB is required for replacement strand synthesis, some loss of pCH8 from donor cells might occur following transfer.

To investigate this hypothesis the stability of pCH8 in the donor strain was measured. KL4330R donors of pCH8 and pLG273 were constructed by conjugation with KL4330 strains carrying these plasmids, with selection for rifampicin and tetracycline resistance. A recombinant plasmid, pLG252, was then introduced into these test strains by transformation. The entry exclusion phenotype specified by this
plasmid (Chatfield et al., 1982) was expected to prevent the conjugative reinfection of any plasmid-free cells, which would mask plasmid loss. However, the mating efficiency of the resulting strains was markedly reduced by the presence of pLG252, as shown in Table 5.1.6. This effect possibly reflects the overproduction of the exclusion determinant in the donor cell (Rees et al., 1987). It was not possible, therefore, to exploit entry exclusion in this experiment. Thus, KL4330R donors harbouring only pCH8 or pLG273 were mated with rifampicin-sensitive, streptomycin-resistant recipients (BW97) at 37°C and matings interrupted after 30 min. The short mating period should prevent any extensive reinfection of plasmid-free cells.

The yield of transconjugants from this mating, selected on medium containing streptomycin and tetracycline, indicated efficient conjugation (Table 5.1.7). Following mating, cultures were plated on medium containing rifampicin to select for the KL4330R donor cells, regardless of whether the conjugative plasmid was present. When 600 rifampicin-resistant colonies from each mating were subsequently tested by patch plating onto medium containing tetracycline, all showed the Tc" phenotype characteristic of the two ColIb plasmids. Thus there was no detectable loss of pCH8 from donor cells after mating and therefore no defect was apparent in the conjugative processing of the mutant plasmid.

As discussed below, to fully interpret the results described above it was necessary to determine whether the donor cells lose viability when mated. Thus, in parallel experiments to the matings described above, donor cells were grown under identical conditions but in the absence of recipient cells. When the numbers of mated and unmated donors were compared no obvious mating-dependent loss of viability of donors carrying the mutant plasmid was apparent (see Table 5.1.8).
TABLE 5.1.6. Effect of the exclusion-specifying recombinant pLC252 on conjugative efficiency of donor strains mated at 37°C for 30 min.

<table>
<thead>
<tr>
<th>Donor Strain</th>
<th>Recipient Strain</th>
<th>Transconjugants formed /ml</th>
<th>Transconjugants formed /donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>KL4330R (pLC273)</td>
<td>BW97</td>
<td>$1.8 \times 10^7$</td>
<td>0.64</td>
</tr>
<tr>
<td>KL4330R (pLC273,pLC252)</td>
<td>BW97</td>
<td>$2.4 \times 10^4$</td>
<td>$8.6 \times 10^{-5}$</td>
</tr>
<tr>
<td>KL4330R (pCH8)</td>
<td>BW97</td>
<td>$1.2 \times 10^7$</td>
<td>0.80</td>
</tr>
<tr>
<td>KL4330R (pCH8,pLC252)</td>
<td>BW97</td>
<td>$2.5 \times 10^4$</td>
<td>$8.3 \times 10^{-5}$</td>
</tr>
</tbody>
</table>
TABLE 5.1.7. Transconjugants formed in 30 min matings at 37°C in matings between exponentially growing and stationary phase KL4330R donors and BW97 recipients.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Donor Growth State</th>
<th>Input Donors /ml</th>
<th>Transconjugants /donor</th>
<th>Donors after 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCH8</td>
<td>exponential</td>
<td>2.1x10^7</td>
<td>3.2x10^7</td>
<td>1.52 4.0x10^7</td>
</tr>
<tr>
<td>pLG273</td>
<td>exponential</td>
<td>2.0x10^7</td>
<td>2.1x10^7</td>
<td>1.05 4.1x10^7</td>
</tr>
<tr>
<td>pCH8</td>
<td>stationary</td>
<td>1.8x10^7</td>
<td>3.7x10^7</td>
<td>2.05 2.0x10^7</td>
</tr>
<tr>
<td>pLG273</td>
<td>stationary</td>
<td>2.1x10^7</td>
<td>5.2x10^7</td>
<td>2.50 2.1x10^7</td>
</tr>
</tbody>
</table>

Results are the mean of three independent experiments.

TABLE 5.1.8 Donor cell viability in 30 min matings at 37°C of KL4330R donors and BW97 recipients.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Input Donors /ml</th>
<th>Transconjugants /donor</th>
<th>Donors after 30 min</th>
<th>Unmated Donors after 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCH8</td>
<td>2.3x10^7</td>
<td>2.3x10^7</td>
<td>1.00 4.6x10^7</td>
<td>4.2x10^7</td>
</tr>
<tr>
<td>pLG273</td>
<td>2.0x10^7</td>
<td>1.7x10^7</td>
<td>0.85 4.2x10^7</td>
<td>4.4x10^7</td>
</tr>
</tbody>
</table>
5.2 Maintenance stability of pCH8.

By analogy with the activity of E. coli SSB, the most likely activity for the plasmid SSB protein is in some aspect of DNA replication. ColIb SSB apparently has no role in conjugal replication. Nevertheless, it may function in the vegetative replication of the plasmid. If this were the case, it might be expected that the mutant plasmid would show some instability as a result of a deficiency in the replication process. The stability of pCH8 was therefore assessed in vegetatively growing cells.

The test strains were BW97 carrying either pCH8 or pLG221 (a ColIbdrd−I derivative containing Tn5). Conjugal reinfection of any plasmid-free segregants was prevented by introducing the recombinant plasmid, pLG252, specifying entry exclusion, into these strains. In addition, the medium was supplemented with 0.01% SDS, which inhibits conjugation by depolymerising the pilin subunits without affecting cell growth (Boulnois et al., 1979). Cultures were grown for approximately 13 generations at densities lower than 2×10⁶ cells per ml, thus maintaining exponential growth. Bacteria were plated on Luria agar and single colonies obtained were tested for expression of the Km⁻ determinant specified by the ColIb plasmids. All 200 colonies tested from each strain retained the Km⁻ marker. The mutant plasmid therefore showed no defect in maintenance.
5.3 Collb does not carry additional sequences homologous to Collb ssb.

The failure to detect a mutant phenotype associated with the insertion in Collb ssb could be explained if Collb-P9 carries a second gene with SSB activity. If such a sequence was present, the results presented in Chapter Two indicate that it clearly does not possess any high degree of homology with the *E. coli* ssb, nor was its product capable of suppressing the temperature or UV sensitivity of an ssb-I strain. Nevertheless, the hypothetical second gene might be homologous to a portion of the plasmid ssb sequence, but not the *E. coli* ssb, and it might suppress certain defects caused by the Collb ssb mutation. A potential candidate is the sog primase gene, since its product also binds to single-stranded DNA (Lanka et al., 1979). Consequently, the Collb ssb sequence, isolated on the 3.95 Clai-SalI fragment of pCH4, was used as a probe in Southern hybridization experiments to determine whether sog showed some homology to Collb ssb.

Fig. 5.3.1 shows that the Collb ssb probe hybridized only to the E1 and S4 fragments from EcoRI and SalI digests of Collbdrd-I, respectively. These fragments are those known to carry the Collb ssb sequence (see Chapter Two). Hybridization was not detected with any other fragments of Collbdrd-I or with a recombinant plasmid, pLG215, which carries the sog gene of Collb (Wilkins et al., 1981). Thus no Collb ssb homologues are present on Collb. However, this finding does not rule out the possibility that Collb encoded an analogue of Collb SSB which was capable of suppressing some of the defects caused by the Collb ssb mutation, but which did not share any significant homology with the plasmid SSB. The plasmid-encoded primase still remained a candidate for such an activity.

In order to determine whether the Collb primase was able to suppress Collb ssb defects, attempts were made to construct a sog ssb double mutant of Collb. Plasmid pLG264 is a Collbdrd-2 derivative
FIG. 5.3.1. Results of Southern hybridization of 3.95 kb Clal–Sall fragment of pCH4 carrying Collb ssb to digests of pLG215 and Collbdrd-I.

A. Agarose gel electrophoresis of pLG215 cut with EcoRI (a), Collbdrd-I cut with EcoRI (b) and pCH4 cut with Clal and Sall (c).

B. Southern blot analysis. Molecular sizes (kb) are indicated.
5.3 Collb does not carry additional sequences homologous to Collb ssb.

The failure to detect a mutant phenotype associated with the insertion in Collb ssb could be explained if Collb-P9 carries a second gene with SSB activity. If such a sequence was present, the results presented in Chapter Two indicate that it clearly does not possess any high degree of homology with the E. coli ssb, nor was its product capable of suppressing the temperature or UV sensitivity of an ssb-1 strain. Nevertheless, the hypothetical second gene might be homologous to a portion of the plasmid ssb sequence, but not the E. coli ssb, and it might suppress certain defects caused by the Collb ssb mutation. A potential candidate is the sog primase gene, since its product also binds to single-stranded DNA (Lanka et al., 1979). Consequently, the Collb ssb sequence, isolated on the 3.95 ClaI-SalI fragment of pCH4, was used as a probe in Southern hybridization experiments to determine whether sog showed some homology to Collb ssb.

Fig. 5.3.1 shows that the Collb ssb probe hybridized only to the E1 and S4 fragments from EcoRI and SalI digests of Collbdrd-I, respectively. These fragments are those known to carry the Collb ssb sequence (see Chapter Two). Hybridization was not detected with any other fragments of Collbdrd-I or with a recombinant plasmid, pLG215, which carries the sog gene of Collb (Wilkins et al., 1981). Thus no Collb ssb homologues are present on Collb. However, this finding does not rule out the possibility that Collb encoded an analogue of Collb SSB which was capable of suppressing some of the defects caused by the Collb ssb mutation, but which did not share any significant homology with the plasmid SSB. The plasmid-encoded primase still remained a candidate for such an activity.

In order to determine whether the Collb primase was able to suppress Collb ssb defects, attempts were made to construct a sog ssb double mutant of Collb. Plasmid pLG264 is a Collbdrd-2 derivative
containing a Tn5 insertion within sog (Merryweather et al., 1986a). Since the marker carried by Tn5 is kanamycin resistance it was not possible to utilize the ssh mutation carried by pCH6 to construct the double mutant by recombination. A derivative of pCH6 was therefore constructed which contained the Tn9-derived chloramphenicol resistance gene from plasmid pYEJ001 (Soberon et al., 1980). Since pCH6 itself carries a Cm" determinant in the vector sequence, it was first necessary to disrupt this gene. This was achieved by destroying the EcoRI site within the Cm" gene by fill-in synthesis and blunt-end ligation to produce plasmid pCH10, which no longer expressed chloramphenicol resistance. Plasmid pCH12 was then constructed by ligating the HindIII fragment of pYEJ001, which carries the Cm" gene, into the HindIII site located within the Km" determinant of the Tn903-derived insertion in pCH10.

Attempts were then made to introduce the mutated Collb ssh gene, tagged with Cm", from pCH12 into plasmid pLG264 by recombination, using the method of Winans et al. (1985) described in Chapter Two. Unfortunately, when introduced into JC7623 Coll", the recBC sbcB strain required for this method, pLG264 apparently lost the Tn5 insertion from within sog. In some cases the Tn5 had probably transposed into another site within the plasmid (Fig 5.3.2). The insertion was apparently stable when the plasmid was introduced into other laboratory strains, including KL4330, BW86 and the recA mutant BW103. Although this suggests that it is the recBC sbcB strain which is responsible for this effect, it should be noted that pLG264 was itself constructed by the method of Winans et al. (1985) using the same recBC sbcB strain. Limitations of time prevented this line of research from being pursued further.
5.3.2. Instability of In5 insertion in plasmid pLG264.

Lanes (a), (b) and (c) show EcoRI digests of three isolates of pLG264 derived from strain JC7623. Lane (d) shows an EcoRI digest of Colibrd-2 and lane (e) shows an EcoRI digest of a pLG264 isolate derived from the recA strain BW103. The marker in lane (f) is a HindIII digest of λcl857 Sam7. The -14kb fragment of pLG264 which corresponds to the E3 fragment of Colib carrying the In5 insertion within sog is apparent in lane (e). In lanes (a), (b) and (c) this fragment is replaced by a -8.5 kb band corresponding to the E3 fragment of Colibrd-2 seen in lane (d). In lane (a) a smaller fragment of -6 kb is apparent which may correspond to a In5 insertion within a smaller EcoRI fragment of Colibrd-2.
5.4 Discussion.

This chapter has described the investigation of the properties of pCH8, a derivative of ColIbdrd-1 carrying a 1.2 kb insertion within ColIb ssb. Plasmid ssb genes appear to be components of conjugation systems, since they are expressed coordinately with the conjugation genes (see Chapter Two). This suggested that ColIb ssb may function in conjugation, and the hypothesis was investigated in tests of conjugative efficiency. However, the mutant plasmid showed little more than a two-fold defect in any of these tests, indicating that ColIb ssb has no major role in either the transfer of plasmid DNA or its processing in the recipient cell. Some of these matings were carried out between ssb-1 donor and recipient cells at 44°C, a temperature at which the SSB-1 protein would be inactivated. Since no major defect in conjugative transfer efficiency was apparent in these matings it can be inferred that the E. coli SSB also has no role in the transfer of plasmid DNA. However, this assumes that the SSB-1 protein is fully inactivated at the high temperature. In addition, a role for this protein in complementary strand synthesis cannot be ruled out.

No loss of the mutant plasmid from donor cells could be detected after mating, indicating that ColIb SSB was not required for replacement strand synthesis in the donor cell. However, ColIb may specify systems analogous to the ccd or hok/sok mechanisms described on F-like plasmids (Gerdes et al., 1986; Ogura et al., 1980) which act to limit the occurrence of plasmid-free segregants by rendering such cells non-viable. It is noted that hok/sok may be in the leader region of F, between oriT and ssb.

The hok/sok system of plasmid R1 (IncFII) relies on the expression of two genes located within the parB region of the plasmid. The hok (host-killing) gene expresses a product which is highly toxic and results in the killing of the host cell. The sok (suppressor of
killing) gene, however, encodes a product which counteracts this hok-mediated killing. It has therefore been proposed that plasmid stability is a result of the killing, by the hok gene product, of any plasmid-free cells that result from the loss of a plasmid carrying the parB locus (Gerdes et al., 1986a,b). The ccd (coupled cell division) system of F is similar in concept. In this case plasmid stability is again a result of the interaction of the products of two plasmid genes, ccdA and ccdB (Ogura and Hiraga, 1983). The ccdB gene encodes an inhibitor of cell division, but ccdB-mediated division inhibition is relieved by the ccdA product. Any plasmid-free segregants resulting from division of a cell containing only one copy of the plasmid are non-viable due to the activity of the ccdB product in the absence of ccdA expression, and form filaments after a few residual divisions (Jaffe et al., 1985).

Any loss of the ColIb ssb mutant plasmid from donor cells might therefore be masked by ccd or hok/sok-like systems encoded by ColIb. Such a mechanism may also invalidate the result which demonstrated that pCH8 was stable in vegetatively growing cells and which was interpreted to indicate that the ColIb ssb product had no role in the maintenance stability of ColIb. It is not known whether ColIb specifies a system analogous to ccd or hok/sok but the stability of the low copy number plasmid suggests that some mechanism may exist to reduce plasmid-free segregant formation. Indirect evidence does exist that ColIb encodes a ccd-like system, since, when plasmid replication is disrupted, both F and ColIb are capable of blocking host cell division (Miki et al., 1988; Monk, 1969; Ogura and Hiraga, 1983). Since ccd and hok/sok both mediate the killing of plasmid-free segregants (Gerdes et al., 1986; Jaffe et al., 1985) the activity of either system might be expected to result in a loss of viability of any plasmid-free segregants occurring as a result of a defect in replacement strand synthesis. However, no loss of donor cell viability was detected after transfer of the ColIb
ssb mutant plasmid. Thus, if Collb does encode a system similar to ccd or hok/sok, this finding suggests that the Collb ssb mutation does not result in any marked plasmid instability.

Plasmid pCH8 showed no marked mutant phenotype with respect to the conjugative process and there is clearly no absolute requirement for Collb SSB. Golub and Low (1986) similarly found no mutant phenotype associated with the disruption of the ssb genes of some other plasmids but they did not describe the nature of these mutations or details of the tests. If Collb SSB has a role in conjugation, it is unlikely to function in complementary strand synthesis in the recipient cell since any requirement would presumably be immediate and before the genes for high level expression are transferred (see Chapter Four).

The possibility that Collb SSB is transported to the recipient cell bound to the transferred strand of DNA has been investigated using a method by which transmitted [35S]methionine-labelled polypeptides can be detected following mating (Merryweather et al., 1986a). No protein of the size predicted for the plasmid SSB was detected in recipient cells after transfer of either plasmid F or drr derivatives of Collb (C. E. D. Rees, personal communication, 1988). Thus, any involvement of the plasmid SSB in conjugation seems more likely to be in the donor cell, possibly to promote replacement strand synthesis.

The possibility that Collb-P9 was derived by ancestral fusion of parts of two conjugative plasmids (Rees et al., 1987; see Chapter One) suggests that the failure to detect any defect in conjugation caused by the Collb ssb mutation may be due to the substitution of Collb SSB function by some other plasmid product. If such a product is encoded by Collb, clearly it is unable to suppress defects in bacterial DNA metabolism caused by the ssb-I mutation. The possibility that Collb encodes a second gene homologous to Collb ssb has been discounted, but a plasmid-encoded product may still be produced which is able to
substitute for ColIb SSB, while being unable to suppress ssb-1 defects. This could be achieved if another protein capable of binding to ssDNA is encoded by the plasmid. The plasmid-encoded DNA primase is able to bind to ssDNA and acts in DNA replication processes during conjugative transfer. This protein may be capable of carrying out the activities for which the plasmid SSB is normally required, possibly an involvement in plasmid replication or in plasmid strand synthesis during conjugation. This possibility remains to be pursued. It should be noted, however, that F SSB similarly has no detectable role in the conjugative process (Golub and Low, 1985) but that plasmid F does not encode a DNA primase detectable by the suppression of the bacterial dnaG3 mutation or the ability of crude cell extracts to stimulate DNA synthesis on M13 DNA in the presence of rifampicin (Lanka and Barth, 1981; Willetts and Wilkins, 1984).
Chapter Six

Properties of an ssh mutant of ColIbdrd-1:
Effect of the mutation on induction of the SOS system.

Introduction

In the previous chapter it is argued that ColIb SSB has no obvious role in the conjugative processing of the transferred plasmid strand in the recipient. However, the experiments described would not have detected a reduced rate of complementary strand synthesis caused by diminished levels of SSB. The accumulation of single-stranded DNA in the recipient as a result of such a defect might act to induce the SOS response (see Chapter One). The hypothetical coating of the transferred DNA with plasmid SSB might reduce the ability of this DNA to act as an inducing signal either directly or by promoting its rapid conversion into double-stranded form. To determine whether a deficiency of ColIb SSB results in increased SOS expression in the recipient cell, the level of \( \lambda \) prophage induction was determined in recipient cells following conjugation with donors of the ColIb ssh mutant, pCH8. The rationale for using this assay was based on the indirect induction phenomenon, the efficient induction of \( \lambda \) prophage which is observed after the transfer of plasmid DNA containing UV photoproducts induced in the recipient cell prior to mating (Borek and Ryan, 1958; Monk, 1969).

It has also been proposed that plasmid psi (plasmid SOS inhibition) genes, described in Chapter One, may have evolved to allow the transfer of single-stranded DNA while avoiding the generation of an SOS inducing signal (Bagdasarian et al., 1980; 1986). It has recently been shown that plasmids carrying ssh genes with homology to F ssh also carry homologous psi genes (see Chapter One). The Psi phenomenon has been primarily studied using the IncFII plasmids R100-1 and R6-5 (Bagdasarian et al., 1980; 1986) which share extensive homology with the leading region of plasmid F and possess ssh genes (E. Golub,
personal communication, 1988; see Chapter One). The psi genes also lie within the leading region, between ssb and oriT.

This chapter also describes experiments designed to investigate whether Collb and its ssb mutant derivative, pCH8, express the Psi phenotype. These experiments used recA441 and recA730 mutant strains in which the induction of SOS genes is released from the requirement for the normal inducing signal. The Psi phenomenon can conveniently be monitored in these strains by measuring the β-galactosidase activity specified by fusions between sfiA, an SOS gene that blocks cell division, and lacZ, the β-galactosidase structural gene [Bagdasarian et al., 1986]. The results show that the mutant plasmid, pCH8, confers a strong Psi+ phenotype whereas the wild type plasmid has no such effect.

6.1 Effect of the Collb ssb mutation on SOS induction in the recipient cell.

Since activated RecA protein promotes the cleavage of λ repressor in addition to the LexA repressor of the SOS genes [see Chapter One], the induction of λ prophage presents a convenient method by which SOS induction can be detected. This approach was used to determine whether the Collb ssb mutation has any effect on SOS induction in a recipient cell following conjugative DNA transfer.

Test strains were first constructed by transferring plasmids pCH8 and pLG273 by conjugation into a λ resistant derivative of KL4330. The Nal+ Tc+ strains obtained were then mated with BW40 Coll+ λ recipients carrying λ prophage. Mating was at 37°C for 90 min, with a three-fold excess of donors which would ensure that a large proportion of potential recipients received the plasmid. The phage titre was assayed after mating. The results obtained (Table 6.1.1) indicate that the level of phage induction was similar whether the transferred plasmid was pLG273 or pCH8. It is apparent that prophage induction after transfer was only slightly greater than the background level obtained.
Table 6.1.1 Phage λ induction following conjugative transfer of plasmids between KL4330λ" donors and BW40 Colr λr (λ+) recipients at 37°C.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Input a Recipients /ml</th>
<th>Transconjugants /ml after 60 min</th>
<th>Phage titre at 90 min /transconjugant</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>--</td>
<td>--</td>
<td>7.7x10 7</td>
</tr>
<tr>
<td>pCH8</td>
<td>1.1x10 8</td>
<td>2.0x10 8</td>
<td>9.1x10 7</td>
</tr>
<tr>
<td>pLC273</td>
<td>1.1x10 8</td>
<td>6.1x10 8</td>
<td>2.5x10 8</td>
</tr>
</tbody>
</table>

a - Mating was carried out with a donor to recipient ratio of 3:1.

Results are the mean of three independent experiments.
In control experiments in which KL4330λa and BW40 Colla λa (λ+)
were
grown together under similar conditions (Table 6.1.1). This indicates
that plasmid transfer does not normally induce SOS functions. However,
it is clear that there is no absolute requirement for the plasmid-
coded SSB in any mechanism which may exist to prevent such induction.

6.2 Effect of Collb ssb mutation on induction of sfiA
in recA441 and recA730 backgrounds.

The expression of the SOS functions in strains harbouring pCH8 or
pLG273 was investigated using GY7221 and GC4597. These strains are λcI
ind1 sfiA::lacZ lysogens of a recA730 and a recA441 strain,
respectively. Test plasmids were introduced into these strains by
conjugation, with selection for the streptomycin resistance of the
bacterial cells and the Tc+ marker carried by the plasmids. Control
strains containing plasmid R100-1 were also constructed. The level of
SOS induction in these strains was determined by assaying the level of
β-galactosidase activity as described in Chapter Eight. Table 6.2.1.
shows that plasmid R100-1 expressed a Psi+ activity in both recA
mutants, in agreement with the previous report (Bagdasarian et al.,
1986). Surprisingly, the results also indicated that plasmid pCH8, in
contrast to pLG273, conferred a marked Psi+ phenotype on the test
strains.

This result was supported by the observation that, when tested in
the recA441 mutant, plasmid pCH9 also specified Psi+ activity (Table
6.2.1). Plasmid pCH9 is a Collb ssb mutant, derived from pLG273, but
isolated independently of pCH8. However, both pCH8 and pCH9 carry the
same pCH6-derived insertion mutation in Collb ssb (see Chapter Two).
In order to confirm that the results described above were not due to
the activity of a truncated protein expressed from the disrupted Collb
ssb gene, the recombinant plasmid, pCH6, which carries the Collb ssb
Table 6.2.1 Effects of plasmids on \textit{sfiA}:\textit{lacZ} expression in \textit{recA441} and \textit{recA730} cells.

<table>
<thead>
<tr>
<th>Plasmid[s]</th>
<th>(\alpha)-galactosidase in strains (Units per mg protein) (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GC4597 (\textit{[recA441]})</td>
</tr>
<tr>
<td>None</td>
<td>1630</td>
</tr>
<tr>
<td>R100d-1</td>
<td>155</td>
</tr>
<tr>
<td>pLG273</td>
<td>1800</td>
</tr>
<tr>
<td>pCH8</td>
<td>313</td>
</tr>
<tr>
<td>pCH9</td>
<td>224</td>
</tr>
<tr>
<td>pCH2</td>
<td>1476</td>
</tr>
<tr>
<td>pCH4</td>
<td>1789</td>
</tr>
<tr>
<td>pCH6</td>
<td>1713</td>
</tr>
<tr>
<td>pACYC184</td>
<td>nt(^b)</td>
</tr>
<tr>
<td>pCH8,pCH4</td>
<td>443</td>
</tr>
<tr>
<td>pCH8,pACYC184</td>
<td>565(^*)</td>
</tr>
<tr>
<td>pCH8,pCH2</td>
<td>386(^*)</td>
</tr>
<tr>
<td>pCH8,pBR322</td>
<td>417(^*)</td>
</tr>
<tr>
<td>pLC2001</td>
<td>1082</td>
</tr>
<tr>
<td>pCH8,pLC2001</td>
<td>401</td>
</tr>
<tr>
<td>pLC221</td>
<td>1891</td>
</tr>
<tr>
<td>pLC272</td>
<td>1919</td>
</tr>
<tr>
<td>pLC273,pCH6</td>
<td>1671</td>
</tr>
<tr>
<td>pLC273,pACYC184</td>
<td>1833</td>
</tr>
</tbody>
</table>

\(^a\) Measured in GC4597 after 1 hr at 42°C and in CY7221 after overnight growth at 30°C. Except where marked \(\text{(*)}\) results are the mean of at least two independent experiments. Results for each experiment were obtained from three replicate assays.

\(^b\) Not tested.
mutation, was also tested. This plasmid did not confer a Psi* phenotype, indicating that the Psi* activity caused by pCH8 is not due simply to the truncated protein produced from the disrupted ColIb ssh gene. Plasmid pCH4 (ssh*) showed no Psi* activity, as expected, but this plasmid was unable to suppress the SOS inhibition caused by the ColIb ssh mutant. Similarly, neither plasmid pCH2, which also carries the cloned ClaI-SaiI fragment containing ColIb ssh* (see Chapter Two), nor plasmid pLG2001, an ssh* plasmid carrying the E1 fragment of ColIb, were able to suppress the Psi* activity of pCH8 in the recA441 mutant. The possibility that pLG273 had suffered a mutation inactivating psi expression, after the construction of the mutant plasmid, can be ruled out since neither pLG221 or pLG272, both ColIb-P9 derivatives, express Psi* activity (Fig. 6.2.1).

Although plasmid pCH6 did not express a Psi* activity, the activity detected with pCH8 might result from the interaction of some other ColIb product with the truncated protein. To investigate this possibility test strains carrying both pLG273 and pCH6 were constructed. The results in Table 6.2.1 show that no SOS inhibition was apparent in the recA441 strain harbouring both plasmids. However, some SOS inhibition was seen in the recA730 strain, indicating that the Psi phenomenon may be due in part to an activity of a protein expressed from both pCH6 and pCH8 as a result of the insertion in ColIb ssh. However, the Psi* effect seen in this strain was not as marked as that observed with pCH8 alone.
6.3 Effect of ColII ssb mutation on SOS induction in recA* cells.

Plasmid R100-1 (IncFII) confers a Psi+ phenotype on recA441 strains at 42°C. However, Bagdasarian et al. (1980) had reported that the presence of this plasmid did not inhibit SOS induction following UV irradiation of rec+ cells. To allow further comparison of pCH8 and R100-1, the effect of the former plasmid on SOS expression in UV-irradiated rec+ cells was also assessed. Plasmids pCH8 and pLG221 were first introduced into GC4415, a rec+ strain carrying a sfiA::lacZ fusion, with selection for the kanamycin resistance conferred by these plasmids. The use of pLG221 as the control plasmid, rather than pLG273, was necessary because GC4415 specifies resistance to tetracycline. The level of SOS induction from these strains was assessed by measuring β-galactosidase expression, as described in the previous section. Test cultures were grown to A600 0.4 and then diluted to A600 0.1. Following UV irradiation of a portion of these test cultures with a dose of 10 J m⁻², the cells were incubated for a further 60 min, after which β-galactosidase levels were assayed. The results shown in Table 6.3.1 indicate that none of the plasmids tested (pCH8, pLG221, pCH4 and pCH6) have any major effect on SOS induction in rec+ cells following UV irradiation. Cells containing the ColII derivatives did show a slightly reduced level of β-galactosidase activity as compared to the plasmid-free cells or those harbouring either of the recombinant plasmids. This may be due to the repair activity of the ColII imp gene products which may remove the SOS inducing signal and reduce the level of SOS expression in these cells. Confirmation of the results described above was obtained by comparing the survival, following UV irradiation at various doses, of W3110 strains harbouring the test plasmids. Fig. 6.3.1 shows that pCH8 did not differ significantly from pLG221 in its effect on the survival of these cells and thus it can be concluded that the presence of the ColII ssb mutation does not have any major
Table 6.3.1 Effects of plasmids on sfiA::lacZ expression in UV-irradiated rec^+ cells.

<table>
<thead>
<tr>
<th>Plasmid(s) present</th>
<th>p-galactosidase in strains (Units per mg protein) a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC4415 Unirradiated</td>
</tr>
<tr>
<td>None</td>
<td>34.0</td>
</tr>
<tr>
<td>pLG221</td>
<td>44.5</td>
</tr>
<tr>
<td>pCH8</td>
<td>61.4</td>
</tr>
<tr>
<td>pCH4</td>
<td>41.4</td>
</tr>
<tr>
<td>pCH6</td>
<td>49.0</td>
</tr>
</tbody>
</table>

a Measured one hour after irradiation with 10 J m^{-2}. Results are the mean of two independent experiments. Results for each experiment were obtained from three replicate assays.
FIG. 6.3.1. Survival of W3110 strains after UV irradiation.

Strains were W3110 (▲), and derivatives harbouring pLG273 (▲) or pCH8 (▼). Results are the mean of two independent experiments.
effect on the UV-induced expression of SOS repair processes.

6.4. Discussion.

Experiments described in the previous chapter did not rule out the possibility that the plasmid-encoded SSB may have a role in the processing of single-stranded plasmid DNA in the recipient cell. If the plasmid SSB does have such a role, a defect in the processing of the transferred single strand would be expected following the transfer of the Collb ssb mutant plasmid, pCH8. The strategy used to investigate this possibility was to screen for \(\lambda\) prophage induction in the recipient cell. Neither the transfer of pLC273 or pCH8 resulted in any great increase in the level of prophage induction in these experiments, indicating that there was no defect in the processing of the transferred DNA to double-stranded form. However, it should be noted that the \(\lambda\) induction assay is relatively insensitive, since the cleavage of \(\lambda\) repressor occurs at approximately one tenth of the rate of LexA cleavage (Little and Mount, 1982). It is also speculative whether the single-stranded DNA which might be accumulated as a result of a defect in processing in the recipient would in fact act as an efficient SOS inducing signal.

The possibility that Collb specifies a \(\psi i\) (plasmid SOS inhibition) gene has been pursued using \(\text{recA}^{441}\) and \(\text{recA}^{730}\) strains carrying \(\text{sfiA}:\text{lacZ}\) fusions. Although no Psi-like phenomenon was apparently specified by the Collb\(\text{drd}-1\) plasmid pLC273, its \(\text{ssb}\) mutant derivative conferred a marked and reproducible Psi\(^+\) phenotype on the \(\text{recA}\) host cells. This suggested that there may be some functional relationship between Collb SSB and a putative Psi protein. However, plasmids R100-1 and R6-5, which possess both \(\text{ssb}\) and \(\psi i\) genes (Bagdasarian et al., 1980; 1986; E. Golub personal communication, 1988) specify a Psi\(^+\) activity without the requirement for the plasmid SSB to
be inactivated (Bagdasarian et al., 1980; 1986).

Plasmid pCH8 did not inhibit SOS induction following UV irradiation of a rec+ cell, initially suggesting that the Psi phenomenon could be a feature of the recA mutations themselves. Similar results were reported by Bagdasarian et al. (1980) with regard to plasmid R100-1. However, the related plasmid R6-5 does inhibit SOS induction in a rec+ strain following nalidixic acid treatment (Bagdasarian et al., 1986). This suggests that SOS induction may occur by different pathways subsequent to different SOS inducing treatments and that the mechanism by which UV irradiation induces SOS functions is not inhibited by the psi products. The possibility that there is more than one pathway for SOS induction has been suggested previously (Lu et al., 1986; Lu and Echols, 1987). RecA protein has been observed to become active for LexA cleavage after binding to UV-irradiated duplex DNA. Thus the ability of RecA protein to directly recognise UV-induced lesions in duplex DNA may provide an alternative mechanism to that provided by single-stranded DNA (Lu and Echols, 1987).

It was not possible to suppress the Psi* phenotype by introducing recombinant plasmids carrying Collb ssh into test strains harbouring pCH8. Thus it appeared that the insertion present within pCH8 had caused a cis-dominant mutation. The dominance of the pCH8-specified Psi* phenotype is apparently not due to the properties of the predicted truncated protein specified by the disrupted Collb ssh gene, since pCH6 did not confer such a phenotype on the recA strains. The possibility that a mutation increasing psi expression had occurred in pLG273 prior to the construction of pCH8 has been discounted since the pLG273 isolate tested as control in the experiments described was derived from the same strain used to construct pCH8.

It also seems unlikely that a second mutation within pCH8 is responsible for the expression of psi since an independently isolated
ssb mutant plasmid (pCH9) also conferred the Psi+ phenotype. The Psi+ phenotype was not apparent when pLG273 and pCH6 were both present in the recA441 test strain. However, some inhibition of SOS-induction was detected when these plasmids were present in the recA730 strain, suggesting that the Psi phenotype may result from an interaction between the truncated protein produced from the disrupted ColIb ssb gene and some other ColIb product. The different results obtained in this case from the two recA mutants may reflect a difference in the nature of the mutant RecA protein produced by the two different strains. However, the results obtained from the temperature sensitive recA441 strain are possibly more reliable since this strain is presumably under less stress during routine culture than the recA730 strain, which constitutively expresses SOS functions at all growth temperatures.

If ColIbdrd-I expresses Psi proteins similar to those of R100-1 and R6-5, it is surprising that the Psi phenotype was apparent only when the ColIb ssb gene was disrupted. Presumably the psi gene carried by pCH8 has been released from its normal regulation as a result of the insertion in ColIb ssb. Sequence data (see Chapter Three) has indicated that ColIb ssb may be part of an operon, and it is possible that the insertion in pCH8 has disrupted the expression of a repressor of psi downstream of the ssb gene. However, this explanation can probably be ruled out since plasmid pLG2001 does not suppress the Psi phenotype conferred by pCH8 and carries, in addition to ColIb ssb, approximately 13 kb of ColIb DNA downstream of the gene. It should be noted that plasmid pLG2001, which does not confer a Psi+ phenotype, carries both ColIb ssb and orIT, and would also be expected to carry psi if the ColIb gene is located in a similar position to that found in the case of plasmid F. However, any downstream repressor of psi would presumably also be present on this plasmid. The most likely explanation
for the results presented in this chapter is that the Psi phenomenon is
due to the overexpression of a \textit{psi} gene, lying downstream of \textit{Collb ssb},
as a result of the insertion carried by pCH8. This is discussed further
in Chapter Seven.
Chapter Seven.

Summary and Discussion.

The work presented in this thesis has demonstrated that plasmid Colb-P9 specifies a single-stranded DNA-binding protein, designated Colb SSB, which shows considerable sequence homology with the SSB proteins specified by plasmid F and by E. coli. The F and Colb ssb genes are also located at similar positions on the two plasmids, mapping on the opposite side of oriT to the defined transfer (tra) genes. The similarity of Colb SSB and E. coli SSB is reflected by the ability of the plasmid protein to suppress defects caused by mutations in the E. coli ssb gene. In contrast to the results published elsewhere (Golub and Low, 1985), the IncP plasmid RP4 does not carry a gene with any extensive homology to F ssb, as determined by DNA-DNA hybridization (see Chapter Two).

The conserved nature of the plasmid ssb sequences suggested that the plasmid SSB proteins may play an important role in the DNA metabolism of the conjugative plasmids which carry them. Golub and Low (1986b) have reported that the ssb genes of plasmids R1 (IncFII), R64 (IncFI) and R100 (IncFII) are coordinately expressed with their respective conjugation genes. The Colb ssb gene has similarly been shown to be expressed coordinately with the Colb tra genes, suggesting that Colb SSB may have a role in conjugation. Thus plasmid ssb genes may have been acquired as components of conjugation systems.

The extensive homology found between the nucleotide and amino acid sequences of the F ssb and Colb ssb genes and proteins was remarkable since the plasmids belong to incompatibility groups sharing very little sequence homology (Falkow et al., 1975; Grindley et al., 1973). Moreover, the conjugation system of Colb is quite distinct from that of F (Rees et al., 1987). However, recent reports by Golub and Low (1985; 1986a) have shown that plasmids from a wide range of different
incompatibility groups carry sequences homologous to the \( \sim 12.9 \) kb leading region of plasmid F, which includes the plasmid ssb. This region of F also includes the \( \psi_{B} \) gene, which lies between ssb and \( \text{ori} \text{T} \) in R6-5 and F (Golub et al., 1988; see below).

By analogy with the role of the \( E. coli \) SSB in the replication of single-stranded DNA phages, the favoured role of Collb SSB is postulated to be in the synthesis of a replacement plasmid strand in the donor or of a complementary strand in the recipient during conjugation. However, since the Collb ssb gene is apparently expressed at a basal level in the absence of \( \text{tra} \) gene expression, Collb SSB may also play a role in the vegetative replication of the plasmid.

It was demonstrated in the work described in Chapter Four that the direction of transfer of Collb is such that the Collb ssb gene is in the leader region of the plasmid, as is the case for F ssb, suggesting that there may be a requirement for the early expression of the plasmid ssb genes in the recipient cell. However, since the genes for high level expression of Collb ssb are transferred late, it is unlikely that there is a requirement for \( \text{de novo} \) synthesis of Collb SSB in the recipient cell during conjugation. Collb SSB therefore probably does not play a role in conjugative DNA metabolism in the recipient, unless the protein is transported with the transferring single-strand, as is the case with the \( \text{sog} \) primase (Chatfield and Wilkins, 1984; Merryweather et al., 1986a; see Chapter One). No transfer of a protein of the size predicted for Collb or \( E. coli \) SSB has been observed (C.E.D. Rees, personal communication, 1988). Thus a role for the protein in the donor cell is suggested, possibly in replacement strand synthesis or to promote the vegetative replication of the plasmid. In either case the plasmid SSB may simply augment the level of the cellular SSB protein.

The direction of transfer of Collb, relative to the \( \text{tra} \) genes, is
the same as that for F, as well as for a number of other conjugative plasmids (Al-Doorl et al., 1982; Coupland et al., 1987; Grinter, 1981; Ippen-Ihler and Minkley, 1986; see Chapter Four). The relative order of gene transfer during conjugation is similar for all transferable plasmids studied so far and this arrangement, in which the tra genes enter the recipient last, may reflect some functional constraint on plasmid structure imposed by the conjugative process. This may relate to a requirement for the expression of the transfer genes in the recipient as a trigger for the active disaggregation of donor and recipient cells following the completion of DNA transfer (Willetts and Skurray, 1980). However, alternative possibilities exist which may explain the observed gene arrangement. The transfer of plasmid DNA requires the unwinding of the two plasmid DNA strands in the donor cell. The unwound plasmid strands will no longer be a suitable substrate for RNA polymerase activity, and therefore expression of plasmid genes would cease. Possibly the transfer genes are required to be expressed in the donor cell for a prolonged period during the act of conjugation, and this could be achieved if the transfer genes are located in the region transferred last during conjugation.

Another possibility is that the late transfer of the tra genes reflects a requirement for the early transfer of replication genes necessary for plasmid maintenance. In the case of plasmids ColIB (IncI1; Rees et al., 1987; Uemera and Mizobuchi, 1982), R100 (IncFII; Womble and Rownd, 1988), and RP4 (IncP; Thomas and Smith, 1987), the replication and incompatibility functions are located at the opposite end of the transfer regions to oriT and are consequently some distance from the leading regions of these plasmids (approximately 35kb in the case of ColIB; see rep/Inc in Fig. 7.1). The non-functional RepFIC region of F (discussed below) is similarly located, although the functional RepFIA and RepFIB regions lie adjacent to and approximately
FIG. 7.1. Map of ColIb-P9 showing the exclusion (exc), single-stranded-DNA-binding protein (ssb), DNA primase (sog), plasmid SOS inhibition (psi; L. Jones and B. M. Wilkins, personal communication, 1988), colicin Ib (cib) and I mutation and protection (imp) genes. The origin of vegetative replication (oriO) and the incompatibility/replication functions (inc/rep) are located in the region which shares homology with the RepFIC probe described in the text (Couturier et al., 1988). The three tra regions are also shown with the origin of transfer (oriT), the approximate location of the pilus genes, and the location of the two positive regulators of the transfer genes (+). Radial lines indicate EcoRI restriction sites. Map data is from Rees et al. (1987), see Chapter One.
12 kb from the leading region, respectively (Willets and Skurray, 1987). However, the overall pattern seen with these four plasmids suggests that there is no general requirement for the replication functions to be transferred early during conjugation.

In order to investigate the role of the plasmid-encoded SSB proteins, a mutant Colb<sup>adr-I</sup> derivative was constructed which carries a Tn<sub>903</sub>-derived Km<sup>r</sup> insertion within the Colb<sup>ssb</sup> gene. This was achieved by first introducing the insertion into the Colb<sup>ssb</sup> gene carried by a recombinant plasmid, and transferring this mutation into a Colb<sup>adr-I</sup> derivative by recombination. Since the sequence of the Tn<sub>903</sub>-derived insertion was known (Oka <em>et al.</em>, 1981), it was possible to use plasmid sequencing techniques to determine the nucleotide sequence of the cloned Colb<sup>ssb</sup> gene (see Chapter Three). The Km<sup>r</sup> insertion proved to be convenient for this sequencing technique since one of the inverted repeat sequences normally present in Tn<sub>903</sub> has been deleted from the commercially available plasmid, pUC4.K, which carries the Km<sup>r</sup> determinant (Vieira and Messing, 1982; Taylor and Rose, 1988). Thus, using an initial primer complementary to the sequence at one end of the insertion, it was possible to determine the nucleotide sequence of the disrupted gene. The truncated protein predicted from these sequencing data to be produced by the mutant Colb<sup>ssb</sup> gene contains only 31 of the amino terminal residues of the Colb SSB protein and therefore does not contain the predicted DNA-binding domain.

The mutant Colb<sup>adr-I</sup> plasmid showed no defect in tests of conjugative efficiency. Thus, despite the coordinate regulation of Colb<sup>ssb</sup> with the transfer genes, the Colb SSB protein apparently has no major role in the transfer of plasmid DNA or in the processing of the transferred DNA in the recipient cell. This is in agreement with results obtained by Golub and Low (1986b), who stated that <sup>ssb</sup> mutants of F and R483 (IncII) did not show any defect in conjugative transfer
or conjugative DNA synthesis. Since ssb-1 donor and recipient cells were used in the experiments described in Chapter Five, the results also suggest that the E. coli SSB is not essential for the conjugative transfer process. However, the possibility remains that the SSB-1 protein may still be active for conjugation processes even at 44°C and may therefore be able to mask any defect caused by the Colb ssb mutation. Due to the essential requirement for the E. coli SSB protein it was necessary, following mating, to plate cells at low temperature (30°C). Since the SSB-1 protein is able to renature at this temperature (Meyer et al., 1979), the possibility that the E. coli protein functions in the conjugative processing of the transferred strand in the recipient cannot be ruled out.

No loss of the Colb ssb mutant from donor cells was detected following transfer, suggesting that the ssb mutation does not cause a defect in replacement strand synthesis in the donor cell. The ssb mutant also showed no defect in maintenance stability, indicating that the Colb SSB is not required for the vegetative replication of the plasmid. However, a defect in either replacement strand synthesis in the donor or in the vegetative replication of the plasmid might be masked if Colb specifies a system which acts to limit the occurrence of plasmid-free segregants, such as hok/sok or ccd, described in Chapter Five.

It has recently been reported that plasmids F and R100 both carry a locus similar to the pari locus of plasmid R1, which encodes the hok and sok gene products (Golub and Panzer, 1988; Womble and Rownd, 1988). The hok (host killing) loci of R1 and F have been shown to specify almost identical polypeptides. In the case of R1 this has been shown to be a membrane-associated lethal protein (Gerdes et al., 1986). The sok (suppressor of killing) locus encodes a non-translated complementary RNA which has been postulated to bind to the hok RNA to suppress
expression of the lethal Hok polypeptide. A third gene, ORF70, identified in F and R1 (Loh et al., 1988) overlaps hok and is also involved in the killing function. The sok product acts by blocking the translation of both ORF70 and hok RNA. 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 the death of plasmid-free segregants results from the eventual translation of the more stable hok RNA in cells which have lost the parB locus (Gerdes et al., 1988).

Expression of the hok gene results in a decrease in the oxygen uptake of the host cell, and a reduction in membrane potential. The mechanism by which the hok gene product results in lethality appears to involve the inhibition of a vital function in the cell membrane due to an interaction between the Hok polypeptide and the membrane (Gerdes et al., 1986b). It should also be noted that overexpression of an E. coli gene, relF, located within the relB operon at 34.4 min on the E. coli map (Bachmann, 1983), has similar effects to those obtained with the R1 hok gene, and leads to the killing of the host cell. The relF gene product shows 40% homology with the Hok polypeptide, and appears to be under the negative control of the neighbouring relB gene product (Bech et al., 1985; Gerdes et al., 1986b). However, in contrast to the effect of the R1 parB locus, the presence of the relB operon does not appear to stabilize oriC minichromosomes, and therefore probably does not act to stabilize the bacterial chromosome. It has been suggested that the relF product acts in a membrane-specific process and that plasmid R1 may have acquired the bacterial relB operon. Subsequently, the function of the plasmid operon has been modified into a mechanism which promotes plasmid stability (Gerdes et al., 1986b).

The parB-like locus of plasmids F and R100 is located within the leading region and lies approximately 0.5 kb from pstB (see below).
between the \textit{psiB} gene and \textit{oriI}. The homology observed between the leading regions of these plasmids and that of ColIb suggests that ColIb may also express a \textit{hok/sok} system. If this is the case, the failure to detect any loss of donor cell viability after mating (see Chapter Five) indicates that there is no loss of the \textit{ssb} mutant plasmid from donor cells. Thus the ColIb SSB does not appear to have an essential role in replacement strand synthesis in the donor cell. Again, it is possible that the \textit{E. coli} SSB protein is able to compensate for any loss of the plasmid protein. However, the plasmid SSB might contribute to efficient maintenance of ColIb, since the activity of a \textit{hok/sok} system would mask any defect in maintenance stability in the experiment described in Chapter Five.

The results of Northern hybridizations have indicated that the \textit{sok} RNA specified by F and R100 is accumulated in unusually large amounts (Golub and Panzer, 1988). However, expression of \textit{sok} was not detected by this method in bacteria carrying the IncI plasmids R64 or R64\textit{der}-\textit{II}, although these plasmids share extensive homology with the F leader region (Golub and Panzer, 1988). Therefore the homology found between the leading regions of many conjugative plasmids is not necessarily continuous, and it is consequently not certain that ColIb does specify a \textit{hok/sok} system. This could easily be resolved by Southern hybridization techniques using the F, R100 or R1 \textit{par} sequences as probe, and by the demonstration that an equivalent region of ColIb is capable of stabilizing naturally unstable multicopy plasmids such as pBR322 or pACYC184, as shown for the F \textit{hok/sok} region (Golub and Panzer, 1988). However, the stability of ColIb (see Chapter Five) suggests that even if the plasmid does not specify a \textit{hok/sok} system it may specify alternative mechanisms for limiting plasmid-free segregants.

In Chapter Six experiments are described which investigated the
possibility that the Collb ssb mutation might lead to an accumulation of ssDNA in a recipient cell following transfer, due to a defect in complementary strand synthesis. This ssDNA might cause an SOS inducing signal (see Chapter One). No induction of λ prophage was detected in recipient cells following transfer, suggesting that there was no defect in complementary strand synthesis. However, it is not clear that an accumulation of ssDNA as described would in fact lead to prophage induction.

An intriguing observation, also described in Chapter Six, is that two independently isolated Collb mutant derivatives of pLG273 conferred a marked Psii* (plasmid SOS inhibition) phenotype on recA441 and recA730 host strains. Such a phenotype is not apparent when pLG273 is present in these strains, indicating that the phenomenon was caused by the disruption of the Collb ssb. It has recently been reported that plasmids which carry ssb genes also carry homologous psi determinants (Colub et al., 1988). The results described above indicate that Collb also carries a psi gene. The finding that the Collb ssb mutant specifies an SOS inhibiting activity may also invalidate the results of the λ induction experiments (see Chapter Six and above), since such a system might prevent induction of λ prophage in these experiments.

It has been speculated that the role of the psi gene products is to allow the transfer of a single-strand of plasmid DNA without inducing an SOS response in the recipient. It was postulated that the Psi activity may result from the binding of the Psi protein to ssDNA, thus competing or interfering with the binding of RecA protein (Bagdasarian et al., 1986). However, Psi protein does not bind to single-stranded DNA, as determined by the elution of mini-cell extracts from a ssDNA-cellulose column (Bailone et al., 1988), and its activity is more likely to result from an interaction with RecA protein to prevent the activation of the RecA coprotease (Devoret et al., 1988;
Since psi proteins do not appear to bind to ssDNA, they are probably not transported with the transferring single strand, unless they form a complex with other transferred proteins. If the psi gene is under coordinate control with the transfer genes, as would be expected if ssh and psi lie within a single operon (see below), then it is unlikely that the gene plays a role in the recipient cell during conjugation, for reasons outlined above. Possibly Psi protein acts in the donor cell to prevent SOS induction by the unwound plasmid strands during transfer.

In addition, Psi proteins may have a role in the vegetative replication of the plasmid. Replication from the P1 origin of mini P1 plasmids has been shown to trigger SOS induction, but the presence of a small EcoR1 fragment derived from P1 prevents this induction (Capage and Scott, 1983). Thus, although capable of basic replication functions, mini P1 plasmids appear to lack a region essential for normal replication. P1 is known to carry a psi gene (Golub et al., 1988) and it is speculated that this gene may be located within the EcoR1 fragment described above. Thus psi genes may have a role in preventing the SOS induction which might otherwise result from plasmid replication. Alternatively, psi genes may have evolved as a safeguard against the induction of SOS functions resulting from aberrant plasmid replication.

However, it is notable that, despite the presence of a psiE gene homologous to that of R6-5, plasmid F does not normally confer a Psi+ phenotype (Golub and Low, 1988). Similarly, no Psi+ activity has been detected with CollbDerrd-I. When present on a multicopy plasmid, however, the F psiE gene does inhibit phage λ and sfiA induction. The Collb Psi activity has only been detected when the Tn903-derived insertion is present within the Collb ssh gene. Furthermore this activity cannot be
suppressed by the presence of an intact Collb ssb gene carried on a recombinant plasmid. Thus the phenomenon is not due to the disruption of the Collb ssb gene per se. Although sequence data suggest that Collb ssb may be located within an operon, the Psi phenomenon does not appear to be due to the failure to express genes downstream of ssb. Possibly the insertion within ssb results in overexpression of the psi gene. Since no strong transcription terminators are present downstream of the Km determinant of the insertion (Oka et al., 1981) it seems likely that readthrough may occur into sequences downstream of Collb ssb. Since Collb ssb appears to be located within an operon, overexpression of downstream genes, possibly including psi, may result.

Since the completion of this work, Dutreix et al. (1988) have shown that the expression of the R6-5 psiB gene is more efficient than that of F. These workers have attributed the ability of R6-5 and R100 to confer a Psi+ phenotype to the presence of a Tn10 insertion, lying between the ssb and psiB genes, which provides a strong promoter (p-OUT) and results in the enhanced expression of the psiB gene. This finding is in agreement with the observation that the F psiB gene confers a Psi phenotype only when expressed at high level.

In the case of F and R6-5, the psiB gene maps close (~2 kb) to ssb, within the leading region of the plasmid, with psi lying between ssb and oriT (Colub et al., 1988; Dutreix et al., 1988). If Collb carries a psi gene located in a similar position, the Tn903 insertion within the ssb gene carried by pCH8 would be orientated such that readthrough from the kanamycin resistance determinant would be in the same direction relative to psi as that postulated in the case of the Tn10 insertion in R6-5 (see Chapter Three). Recently the prediction that Collb carries a psi gene within the leading region has been confirmed by Southern hybridization techniques (L. Jones and B. M. Wilkins, personal communication, 1988). The Collb psi gene lies
approximately 2 kb from \textit{ssb}, between \textit{ssb} and \textit{oriT}, and is therefore located in a position which corresponds to that of the F, R100 and R6-5 \textit{psi} genes. These observations therefore support the argument that the \textit{Psi} phenomenon detected with the Collb \textit{ssb} mutant is due to readthrough from the \textit{Tn903} insertion.

If \textit{Psi} activity is only evident when the genes responsible are expressed at high level, either on a recombinant plasmid or as a result of a strong upstream promoter, it seems questionable whether the normal role of the \textit{Psi} proteins is in the inhibition of SOS expression. Nevertheless, the effect of the proteins on SOS activities suggests that they may have interactions with \textit{RecA} or other cofactors involved in the activation of \textit{RecA} coprotease activity or in other \textit{RecA}-mediated activities. It is interesting that \textit{Psi} activity is apparent in the \textit{recA} strains described above (Bagdasarian \textit{et al.}, 1980; 1986; see Chapter Six), and after nalidixic acid treatment of a \textit{rec}^+ strain, but not after UV irradiation of a \textit{rec}^+ strain (Bagdasarian \textit{et al.}, 1986). This suggests that the inducing signal generated after UV irradiation differs from that generated after other SOS inducing treatments, and from the signal normally recognised by the mutant \textit{RecA} proteins. The possibility that there is more than one pathway for SOS induction has been suggested previously (Lu \textit{et al.}, 1986; Lu and Echols, 1987), and may relate to the ability of \textit{RecA} protein to directly recognise lesions in duplex DNA (Lu and Echols, 1987; see Chapter Six).

The finding that the Collb \textit{SSB} protein apparently has no major role in conjugation or for maintenance of the plasmid suggested that Collb might specify an analogue of Collb \textit{SSB}. The functional analysis of the Collb conjugation system had already lead to the proposal that Collb may carry parts of two ancestral conjugative systems (Rees \textit{et al.}, 1987). A number of conjugation genes, possibly including the plasmid \textit{SSB}, may therefore have been duplicated in Collb.
Recent techniques of replicon typing have allowed the relationships between plasmids to be elucidated by DNA-DNA hybridization using probes derived from the basic replicons of plasmids from a number of incompatibility groups (Couturier et al., 1988). These analyses have shown that plasmids from incompatibility groups FI, FII, FIV, FVI, II, B/O, K, SI and SII belong to a single group of plasmids carrying sequences homologous to part of one of the basic replicons of the IncFI plasmids, designated RepFIC (Table 7.1). Thus these plasmids apparently carry common ancestral sequences. It is the members of this group of plasmids which carry ssb and psi genes (Table 7.1) and show homology with the leading region of plasmid F (Golub and Low, 1985; 1986b). A large number of plasmids which were previously thought to be quite distinct therefore appear to share a region of homology which includes RepFIC-like replication functions and a leading region including the ssb and psi genes. These plasmids also characteristically, although not uniquely, specify flexible pili, thought to be required for mating in liquid (Bradley, 1984; see Table 7.1). With the exception of IncT plasmids, plasmids which do not show homology to the RepFIC probe lack ssb and psi genes (Table 7.1).

Plasmids exemplified by members of the P, M, and U incompatibility groups belong to a group of plasmids which specify DNA primases and rigid pili (which mediate surface-obligatory conjugation [Bradley, 1984]), but do not possess ssb or psi genes or show homology with the RepFIC probe. IncII, IncB and IncK plasmids appear to have characteristics of both of the groups described above. These plasmids encode rigid pili, specify DNA primases and also carry ssb, psi and RepFIC sequences. This suggests that the tra region of these plasmids may be derived from an ancestral fusion of parts of two conjugative systems. This is particularly clear in the case of ColII, since it is known that the expression of the tra genes of this plasmid requires the
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- Determined by hybridization with F plasmid RepFIC region probe (see text; data from Couturier et al., 1988).
- Data for pilus type from Bradley (1980a,b; 1983; 1984).
- Determined by hybridization with F ssb probe and by suppression of the ssb-1 mutation of E. coli (data from Golub and Low, 1985).
- Determined by hybridization with psl gene of plasmid R6-5 (data from Golub et al., 1988).
- Data for primase activity from Lanka and Barth (1981).
activity of two positive regulators (Rees et al., 1987). In addition, the arrangement of the tra genes on Colb (Fig. 7.1) suggests that the Tra1 region may represent part of an F-like conjugation system, including the ssb and psi genes, which has been disrupted by the insertion of the Tra2 and Tra3 regions. The Tra2 region and orfT site may be derived from an IncP or IncM-like system, since this region includes the genes for synthesis of the rigid II-pili, which resemble the rigid pili of the M and P conjugation systems, as well as the gene encoding the sog DNA primase (Rees et al., 1987). Colb therefore shows resemblances not only to the conjugation system of F, in that it possesses ssb, psi and RepFIC sequences and specifies flexible pili, but also to those specified by IncM and IncP plasmids (Rees et al., 1987). Plasmids of the IncM, N, P, U, and W groups share phenotypically similar conjugation systems (Bradley 1980), and all lack F ssh homology (Golub and Low, 1985; see Chapter Two and Table 7.1). Possibly plasmids lacking an F ssh homologue specify analogues of SSB.

The failure to detect any change of phenotype with regard to conjugative processes or plasmid stability associated with the Colb ssh mutation might therefore be explained if Colb specified a protein which was able to suppress defects caused by this mutation. Colb does not carry any additional sequences homologous to Colb ssh, as determined by DNA-DNA hybridization studies (Chapter Five). However, the postulated partially duplicated conjugation system of Colb may specify an SSB analogue, as well as Colb SSB. A candidate for such a protein might be the sog DNA primase, which is required for conjugative DNA synthesis, is able to bind to ssDNA and is transmitted to the recipient with the transferring plasmid strand (Merryweather et al., 1986b).

Not all plasmids which lack ssh genes apparently specify DNA primases. However, since the assay for plasmid primases requires either
the suppression of the bacterial dnaS3 mutation, or priming activity on M13 DNA, it is possible that these plasmids may specify plasmid-specific primases which are not detectable by these assays. It is therefore possible that plasmid DNA primases are able to carry out the functions performed by the SSB proteins specified by other plasmids, such as F and Collb. In the case of Collb, which specifies both proteins, the activities of the plasmid SSB may consequently be redundant.

A mutation in the F ssb gene does not have any detectable effect on conjugation processes (Golub and Low, 1986b). However, plasmid F does not specify a DNA primase, at least as detected by the techniques described above. It is therefore possible that both F and Collb, as well as those plasmids which do not carry ssb sequences, may specify other proteins which may be able to carry out the same functions as an SSB protein, while showing little homology to SSB.

Knowledge of the amino acid sequences of the E. coli and F SSB proteins allowed a survey of computer databanks to be carried out to identify proteins showing homology to these SSB proteins. Although the IncP plasmid RP4 does not carry an ssb gene, plasmid RK2, which is indistinguishable, on the basis of restriction or heteroduplex analysis, from RP4 (Thomas and Smith, 1987), does carry a sequence (trfA) which encodes a polypeptide showing approximately 28% direct homology with the F, Collb and E. coli SSB proteins, as well as possessing many conservative amino acid substitutions (see Chapter Three). This RK2 polypeptide is encoded by open reading frame one [ORF1] of the trfA operon, contains 116 amino acids and appears to correspond to the amino terminal region of the SSB proteins, which includes the DNA binding domain.

The other two known polypeptide products of trfA are essential for the initiation of replication at oriO, and bind to DNA. However, the
ORF1 polypeptide may be responsible for the KilD* phenotype, which prevents plasmid maintenance in the absence of the kor (kil-override) gene, trfB, which maps approximately 18 kb from trfA (Thomas and Smith, 1987). The IncP kil and kor genes may encode products required for partitioning functions or alternatively may constitute a system similar to the hok/sok or ccd systems, described in Chapter Five (Thomas and Smith, 1987).

The homology with the DNA binding region of the SSB proteins suggests that the trfA ORF1 polypeptide may be able to bind ssDNA, but the absence of regions corresponding to the carboxyl terminal third of SSB suggests that its role differs from that of the SSB proteins. Nevertheless, it is possible that this protein may be able to carry out some of the functions of a plasmid SSB.

In conclusion, despite the location of the ColIb ssh gene outside the currently defined transfer regions, the coordinate regulation of the gene with the transfer functions suggests that the plasmid SSB protein plays some role in the conjugative transfer process. By analogy with the role of the E. coli SSB, it might be expected that the plasmid protein would participate in DNA synthesis, either during conjugation or in the vegetative replication of the plasmid. The fact that the genes for high level expression of ssh are transferred late during conjugation, and the failure to detect transfer of the plasmid SSB (C. E. D. Rees, personal communication, 1988) favours a role for the protein in the donor. Thus it seems most likely that the protein acts to promote replacement strand synthesis in the donor cell.

However, the work described in this thesis indicates that there is no absolute requirement for the ColIb ssh gene either in conjugative DNA processing or in the stable maintenance of the plasmid. It has been noted, however, that the effect of an ssh mutation may be masked by other plasmid systems, such as hok/sok or psi. In addition, the
possibility that the plasmid specifies an analogue of Collb SSB cannot be ruled out.

It has recently become apparent that many plasmids which were previously thought to be quite distinct in fact probably carry common ancestral sequences. It seems likely that these plasmids are derived from a single ancestral plasmid which carried the RepFIC sequence. This plasmid at some stage acquired a conjugation system which probably included the flexible pili, ssb and psf genes. Thus the presence of homologous genes located in the same relative positions on both F and Collb is not surprising, since they are probably the remnants of a common ancestral system. Subsequently these plasmids have diverged following the acquisition of additional DNA sequences. In the case of Collb, this has apparently included part of a second conjugation system, presumably derived from an IncP or IncM-like plasmid, which includes the genes for the rigid pili and a DNA primase.

Possibly the ssb gene played a significant role in the conjugation or replication systems of an ancestral plasmid, but is no longer required by many plasmids, including Collb, due to the duplication of plasmid genes with similar activities. The plasmid SSB may, however, promote conjugation or plasmid maintenance processes under certain environmental conditions, or in certain host strains which are not recognised in standard laboratory experiments. In addition, the functional similarity between the E. coli and plasmid ssb products suggests that, whatever the role of the plasmid SSB, the protein probably also acts to augment the level of the cellular protein, and possibly has marginal effects on many aspects of DNA metabolism. It may be for these reasons that the ssb gene is retained by many plasmids.
Chapter Eight

Materials and Methods.


Bacteriophages, plasmids and *Escherichia coli* K-12 derivatives used, their genotypes and source are described in Table 8.1. Plasmid pLG273 was constructed by infection of a strain containing ColIb-P9*d*-*d*-*I* with phage λ840 which carries the Tn10oct4-HH104 element described by Way et al., (1984), with selection for tetracycline resistance and subsequent screening for loss of colicin Ib activity. pLG272 was isolated by the same procedure, involving λ::Tn5 infection of a ColIb-P9 containing strain and selection for kanamycin resistance (N. Smith, personal communication, 1987). Other plasmid constructions are described in the text. For the purposes of publication (J. Bacteriol, in press) some of these plasmids have been allocated names different from those described in this thesis. These plasmids are pLG283 (equivalent to pCH1), pLG284 (=pCH4), pLG286 (=pCH6), pLG288 (=pCH8) and pLG289 (=pCH14).

Bacterial strain KL4330R is a spontaneous rifampicin-resistant derivative of KL4330, isolated by plating 10ml of cells (A660 0.35, Bausch and Lomb, Spectronic-20 spectrophotometer), concentrated to a volume of 0.1 ml, onto Luria agar plates containing rifampicin at 15 µg ml⁻¹. KL4330 is in fact equivalent to strain KL450 (Kolodkin et al., 1983), sent by Dr. E. Golub with a new designation.

A spontaneous colicin Ib resistant derivative of KL4330 was isolated by the following procedure. A ColIb-P9*d*-*d*-*I* containing strain of *E. coli* was plated onto Luria agar. Cells were incubated overnight, the bacteria scraped off and the plates treated with CHCl₃ to kill any remaining cells. 0.1 ml of a ten-fold concentrated overnight culture (A660 2.0) of KL4330 was then plated onto these colicin Ib containing plates. Following incubation overnight at 30°C, colicin Ib resistant colonies were picked from these plates.
Table 8.1.1. Bacterial strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or description</th>
<th>Source or Reference</th>
</tr>
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<tbody>
<tr>
<td>E. coli K-12</td>
<td>proA2 thr leu-8 arg-3 his-4 thi trp lacY galK ara-14 mil-1 xyl-5 uvrB5 rpsL31</td>
<td>B. M. Wilkins et al., (1971)</td>
</tr>
<tr>
<td>BW39</td>
<td>tdk-1 uvrB5 rpsL31 tsz-33 proA2 leu-8 thr-4 arg-3 his-4 thi ara-14 mil-1 xyl-5 gal-2 lac-1</td>
<td>Wilkins et al., (1979)</td>
</tr>
<tr>
<td>BW85</td>
<td>thyA deoB rpsL leu cir</td>
<td>Bougnois et al., (1979)</td>
</tr>
<tr>
<td>BW86</td>
<td>dnaG3 thyA deoB rpsL Δ(chiA-uvrB) leu cir</td>
<td>Bougnois and Wilkins (1979)</td>
</tr>
<tr>
<td>BW97</td>
<td>leu thyA deoB rpsL cir ColI Δ(chiA-uvrB) mal</td>
<td>Bougnois and Wilkins (1979)</td>
</tr>
<tr>
<td>BW103</td>
<td>deoB rpsL leu cir recA1</td>
<td>Merryweather et al., (1986b)</td>
</tr>
<tr>
<td>BW105</td>
<td>C600 λWJB360::pLG253</td>
<td>This work.</td>
</tr>
<tr>
<td>BW106</td>
<td>C600 λWJB360::pCB104</td>
<td>This work.</td>
</tr>
<tr>
<td>BW107</td>
<td>C600 λWJB360::pLG254</td>
<td>This work.</td>
</tr>
<tr>
<td>BW108</td>
<td>C600 λWJB360::pCB105</td>
<td>This work.</td>
</tr>
<tr>
<td>C600</td>
<td>thr-1 leu-6 thi-1 sup644 lacY1 fhuA21</td>
<td>Bachmann (1987)</td>
</tr>
<tr>
<td>CB123</td>
<td>thr-1 leu86 thi-1 lacY1 galK2 ara-14 xyl-5 mil-1 proA2 his-3 arg-3 tsz-33 sup644 rpsL31</td>
<td>C. Boyd</td>
</tr>
<tr>
<td>CC4597</td>
<td>thr leu his arg6 proA lac gal rpsL recA41 (λclindI sfiA::lacZ+)</td>
<td>Huisman and D'Ari (1983)</td>
</tr>
<tr>
<td>CC4415</td>
<td>rec'- thr leu purD (ura)-trp::Mu his Δlac gal malB rpsL sfiA::Mud(AP lacZ+)</td>
<td>D'Ari and Huisman (1981);</td>
</tr>
<tr>
<td>CY7221</td>
<td>recA30 Δlac sfiA211 (λclindI sfiA::lacZ+)</td>
<td>R. Devoret</td>
</tr>
<tr>
<td>KL4330</td>
<td>F- ssb-1</td>
<td>Kolodkin et al. (1983)</td>
</tr>
<tr>
<td>JC7623</td>
<td>recB21 recC22 sbcB15</td>
<td>Kushner et al. (1971)</td>
</tr>
<tr>
<td>RU2537</td>
<td>pro met recA56 mal Tn1723</td>
<td>Ubben and Schmitt, (1986)</td>
</tr>
<tr>
<td>W3110</td>
<td>prototrophic</td>
<td>Bachmann (1972)</td>
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</table>

Genetic symbols are defined in Bachmann (1983).
## Table 8.1.2. Plasmids.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype or description</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ColIb-P9</td>
<td>IncI1, ssb* repressed for transfer</td>
<td>Laboratory stock.</td>
</tr>
<tr>
<td>ColIb-P9</td>
<td>IncI1, ssb* derepressed for transfer</td>
<td>Laboratory stock.</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Cm&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Chang and Cohen (1978)</td>
</tr>
<tr>
<td>pBR328</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Soberon et al. (1980)</td>
</tr>
<tr>
<td>pCB104</td>
<td>λ&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt; lacZ&lt;sup&gt;+&lt;/sup&gt; Δcl</td>
<td>C. Boyd</td>
</tr>
<tr>
<td>pCB105</td>
<td>λ&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt; lacZ&lt;sup&gt;+&lt;/sup&gt; Δcl</td>
<td>C. Boyd</td>
</tr>
<tr>
<td>pCH1</td>
<td>pBR328 O[ColIb ssb&lt;sup&gt;+&lt;/sup&gt;, S&lt;sup&gt;al&lt;/sup&gt;1 10.1 kb Δ 2.35 kb], Ap&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>pCH2</td>
<td>pBR328 O[ColIb ssb&lt;sup&gt;+&lt;/sup&gt;, C&lt;sup&gt;laI&lt;/sup&gt;-&lt;sup&gt;S&lt;/sup&gt;&lt;sup&gt;al&lt;/sup&gt;1 6.3 kb Δ 2.35 kb], Ap&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pCH4</td>
<td>pACYC184 O[ColIb ssb&lt;sup&gt;+&lt;/sup&gt;, C&lt;sup&gt;laI&lt;/sup&gt;-&lt;sup&gt;S&lt;/sup&gt;&lt;sup&gt;al&lt;/sup&gt;1 6.3 kb Δ 2.35 kb], Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pCH5</td>
<td>pCH4&lt;sup&gt;+&lt;/sup&gt;[aphA-I, P&lt;sup&gt;stl&lt;/sup&gt; 1.2 kb], Cm&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pCH6</td>
<td>pCH4&lt;sup&gt;+&lt;/sup&gt;[aphA-I, P&lt;sup&gt;stl&lt;/sup&gt; 1.2 kb], Cm&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pCH8</td>
<td>pLG273&lt;sup&gt;+&lt;/sup&gt;[aphA-I, P&lt;sup&gt;stl&lt;/sup&gt; 1.2 kb], Km&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>pCH9</td>
<td>pLG273&lt;sup&gt;+&lt;/sup&gt;[aphA-I, P&lt;sup&gt;stl&lt;/sup&gt; 1.2 kb], Km&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pCH10</td>
<td>pCH6&lt;sup&gt;+&lt;/sup&gt;Cm&lt;sup&gt;r&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>pCH12</td>
<td>pCH10&lt;sup&gt;+&lt;/sup&gt;aphA-I&lt;sup&gt;+&lt;/sup&gt;[Cm&lt;sup&gt;r&lt;/sup&gt;, ]</td>
<td>This work</td>
</tr>
<tr>
<td>pCH14</td>
<td>pLG339 O[ColIb ssb&lt;sup&gt;+&lt;/sup&gt;, C&lt;sup&gt;laI&lt;/sup&gt;-&lt;sup&gt;S&lt;/sup&gt;&lt;sup&gt;al&lt;/sup&gt;1 6.3 kb Δ 2.35 kb], Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>pCRS4</td>
<td>pBR328 O[ColIb ssb&lt;sup&gt;+&lt;/sup&gt;, S&lt;sup&gt;al&lt;/sup&gt;1 10.1 kb], Ap&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>C. E. D. Rees</td>
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<td>pLG221</td>
<td>ColIb-P9&lt;sup&gt;+&lt;/sup&gt;drd-1&lt;sup&gt;+&lt;/sup&gt; cib::Tn5, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>BouInois (1981)</td>
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Table 8.1.2. Plasmids (continued).

<table>
<thead>
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<th>Plasmid</th>
<th>Genotype or description</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLG252</td>
<td>pBR325 Δ[ColIb 3.5 kb, Ap' Tc']</td>
<td>Chatfield et al. (1982)</td>
</tr>
<tr>
<td>pLG253</td>
<td>pCB104 Δ[ColIb oriT, EcoRI 1.55 kb], Cm'</td>
<td>This work</td>
</tr>
<tr>
<td>pLG254</td>
<td>pCB105 Δ[ColIb oriT, PstI 1.55 kb], Cm'</td>
<td>This work</td>
</tr>
<tr>
<td>pLG264</td>
<td>ColIb-P9drd-2 sog::Tn5, Km'</td>
<td>Merryweather et al. (1986a)</td>
</tr>
<tr>
<td>pLG272</td>
<td>ColIb-P9 cib::Tn5 ssb-, Km'</td>
<td>N. Smith</td>
</tr>
<tr>
<td>pLG273</td>
<td>ColIb-P9drd-1 cib::TnI0 ssb+, Tc'</td>
<td>This work</td>
</tr>
<tr>
<td>pLG274.2</td>
<td>ColIb-P9drd-1 tra::TnI723 ssb+R, Km'</td>
<td>Rees et al. (1987)</td>
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<td>pLG276.3</td>
<td>ColIb-P9drd-1 tra::Tn5 ssb+, Km'</td>
<td>Rees et al. (1987)</td>
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<td>pLG2001</td>
<td>pBR328 Δ[ColIb ssb+, EcoRI 20.25 kb], Ap' Tc'</td>
<td>Merryweather et al. (1987)</td>
</tr>
<tr>
<td>pLG2008</td>
<td>pBR328 Δ[ColIb oriT, PstI 1.55 kb], Cm' Tc'</td>
<td>Rees et al. (1987)</td>
</tr>
<tr>
<td>pYEJ001</td>
<td>Ap' Cm' Tc'</td>
<td>Soberon et al. (1980)</td>
</tr>
<tr>
<td>RP4</td>
<td>IncP</td>
<td>Laboratory stock</td>
</tr>
</tbody>
</table>

Table 8.1.3. Bacteriophage.

<table>
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<th>Phage</th>
<th>Genotype or description</th>
<th>Source or Reference</th>
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<tr>
<td>λWJB360</td>
<td>λcI1288min5Δ[srI1-2] srlA3''srI4''srI5''</td>
<td>Murray et al., (1977)</td>
</tr>
<tr>
<td>λB40</td>
<td>λgt7-his cI1288Par80min5 Tn10d1d-T1104</td>
<td>Way et al., (1984)</td>
</tr>
</tbody>
</table>
Spontaneous \( \lambda \) resistant mutants of KL4330, and of BW40 were isolated by spotting 50 \( \mu l \) of a suspension of \( \lambda_{vir} \) \((1 \times 10^7 \text{ cfu ml}^{-1})\) onto a Luria agar plate. Following incubation at 30°C for 36 hours, colonies resistant to lysis were picked from these plates.

8.2. Media and radiochemicals.

8.2.1. Media.

Bacteria were routinely grown in Luria broth \((10 \text{ g l}^{-1} \text{ Difco Bacto tryptone; } 5 \text{ g l}^{-1} \text{ Difco yeast extract; } 5 \text{ g l}^{-1} \text{ NaCl; pH 7.0})\), with aeration and on Luria agar plates \((\text{as Luria broth plus } 17 \text{ g l}^{-1} \text{ Difco Bacto agar})\).

Minimal media consisted of M9 salts \((42 \text{ mM Na}_2\text{HPO}_4, 22 \text{ mM KH}_2\text{PO}_4, 18 \text{ mM NH}_4\text{Cl, } 8.5 \text{ mM NaCl), 0.1 mM CaCl}_2, 1\text{mM MgSO}_4, 3 \mu\text{M thiamine HCI, } 22\text{mM glucose plus } 15 \text{ gm l}^{-1} \text{ agar (Oxoid No. 2). Appropriate amino acids were added at } 20 \mu\text{g ml}^{-1} \text{ final concentration, except leucine at } 40 \mu\text{g ml}^{-1} \text{ when testing for auxotrophic mutants. SGC (salts-glucose-casamino acids medium) consists of salts as for minimal agar plus } 22\text{mM glucose and } 0.2 \text{ g l}^{-1} \text{ Casamino acids. }}\)

Appropriate antibiotics were added to the medium for plasmid containing strains at the following concentrations: ampicillin \((\text{Ap}), 100\mu\text{g ml}^{-1}\); chloramphenicol \((\text{Cm}), 25\mu\text{g ml}^{-1}\); kanamycin \((\text{Km}), 50\mu\text{g ml}^{-1}\); nalidixic acid \((\text{Nx}), 25\mu\text{g ml}^{-1}\); rifampicin \((\text{Rf}), 25\mu\text{g ml}^{-1}\); streptomycin \((\text{Sm}), 200\mu\text{g ml}^{-1}\); and tetracycline \((\text{Tc}), 7.5\mu\text{g ml}^{-1}\), unless otherwise stated.
8.2.2. Radiochemicals.

Radiochemicals were supplied by Amersham International. 

$[^\alpha-\text{32P}]d\text{CTP}$: specific activity was 11.1 TBq mmol$^{-1}$, at a concentration of 3.7 MBq ml$^{-1}$.

$[^\alpha-\text{35S}]d\text{ATP}$: specific activity was 44 TBq mmol$^{-1}$, at a concentration of 370 MBq \mu l$^{-1}$.

8.3. Phenotypic characterization of bacterial strains.

8.3.1. Determination of growth requirements and resistances.

Growth requirements were assessed by streaking or plating dilutions of bacterial cultures onto minimal media plates containing appropriate amino acids, at the concentrations given above. The ability to utilize galactose was assessed by replacing glucose in the minimal media with 0.2 % galactose.

Strains carrying antibiotic resistance markers were tested by plating onto Luria agar containing the appropriate antibiotics.

8.3.2. Production of colicin Ib.

For mass screening for loss of colicin Ib production, replica plates were made onto Luria agar and incubated overnight. One of each set of plates was then treated with chloroform vapour for 20 min to kill the test colonies, and the chloroform evaporated from these plates for 30 min. The treated plates were then overlayed with 3.5 ml soft nutrient agar containing 0.1 ml of a twentieth dilution of an overnight culture of a colicin Ib sensitive indicator strain. Plates were incubated at 37°C and colicin Ib producing strains showed a clear region of killing around the colony. Testing of individual strains was similar except that 20 - 30 \mu l of the test strain was spotted onto the Luria agar plate.
8.3.3. Sensitivity to bacteriophage.

Sensitivity to bacteriophage was assessed by spotting 10 μl of a dilution of phage suspension on to 0.1 ml of an exponential phase culture (A660 0.35) spread in 3.5 ml soft nutrient agar onto a Luria agar plate. Sensitivity was indicated by the presence of plaques.

8.3.4. Suppression of ssp-1.

Suppression of temperature sensitivity 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. UV survival was measured using overnight cultures diluted to A660 of 0.35. A further 10 fold dilution in phosphate buffer was irradiated at 0.5 J m⁻² sec⁻¹. Samples were taken after appropriate doses, plated onto Luria agar plates and incubated at 30°C, with appropriate selection for plasmid markers. Colonies were counted and survival expressed as the fraction of colony forming units as compared with a similarly treated but unirradiated sample.

8.3.5. 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. Overnight cultures were diluted to A660 0.05 and, in the case of the recA730 strains (GY7221) grown to A660 0.35 at 37°C with aeration and assayed for β-galactosidase activity. The recA441 strains (GC4597) were grown to A660 0.35, at 30°C, then transferred to 42°C, adenine added (0.5 mM) and the cultures incubated for a further 1 hour with aeration.

Prior to assaying β-galactosidase activity the A660 of the cultures was measured using a Unicam spectrophotometer. The assay was set up in 2.0 ml volumes. Depending on the level of activity expected, tubes were set up containing either 0.2 ml culture; 1.8 ml Z-buffer (60
mM Na₂HPO₄; 40 mM NaH₂PO₄; 10mM KCl; 1 mM MgSO₄) or 1.0 ml culture and 1.0 ml Z-buffer. 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, with gentle shaking, at 37°C for 1.5 to 2 hours. Once the toluene had evaporated tubes were transferred to 28°C and 0.4 ml ONPG (ortho-nitryl-phenyl-guanosine) added. When the ONPG indicator had changed to a pale yellow the reaction was stopped by the addition of 1.0 ml 1M NaCO₃ and the time taken for the colour change recorded. The A₄₂₀ and background A₅₆₀ readings were then taken using the Unicam spectrophotometer. The specific activity of β-galactosidase was then calculated using the following formula:

\[
\text{Specific Activity} = \frac{([A_{420} - (1.75 \times A_{560})] \times r)}{[0.0075 \times t]} - \frac{v}{(A_{560} \times 0.227)}
\]

where
- \( t \) = time for reaction (seconds)
- \( r \) = reaction volume (3.4 ml)
- \( v \) = sample volume (0.2 or 1.0 ml)
8.4. DNA manipulations.

8.4.1. Small-scale plasmid DNA preparations.

A modification of the alkaline sodium dodecyl sulphate method of Birnboim and Doly (1979; Ish-Howowicz and Burke, 1981) was used for all routine preparation of plasmid DNA, for restriction analysis and for cloning procedures. 1.6 ml of an overnight culture of cells was centrifuged in an MSE microfuge for 1 min, and the supernatant carefully removed. The pellet was resuspended in lysis buffer (100 µl of 25 mM Tris-HCl, pH 8; 10 mM EDTA, 50 mM sucrose) plus 50 µl lysozyme (50 mg ml\(^{-1}\)). Cells were incubated on ice for 5 min prior to the addition of 200 µl alkaline SDS (0.2 M NaOH; 1% SDS). This was mixed gently and incubated on ice for a further 5 min. 150 µl of 3 M potassium acetate solution (pH 4.8) were then added, mixed in vigorously and incubated on ice for 5 min. The lysate was centrifuged for 2 min in an MSE microfuge and the supernatant transferred to a clean Eppendorf tube containing an equal volume of phenol mix (100 g phenol [Fisons]; 0.1 g 8-hydroxyquinoline [Sigma]; 4 ml isoamylalcohol [Fisons] in 100 ml chloroform [Fisons] stored under 10 mM Tris-HCl at 4°C). The tube was mixed well and centrifuged for 3 min. The phenol extraction was repeated, with a 1 min spin, and the supernatant transferred to a clean tube containing an equal volume of a 24:1 chloroform:isoamylalcohol mixture, mixed well and centrifuged for 3 min. The chloroform extraction was then repeated once more with a 1 min spin and the upper layer carefully removed. The DNA was precipitated by adding a tenth volume 2M sodium acetate (pH 5.6), and two volumes absolute ethanol. Following incubation at room temperature for 5 min the DNA was pelleted for 3 min in an MSE microfuge. The pellet was washed twice with 70% ethanol, vacuum desiccated, resuspended in 50 µl distilled water and stored at 4°C.

Plasmid DNA for nucleotide sequence determination was prepared by
Table 8.4.1. Standard molecular weight DNA markers (kilobases).

<table>
<thead>
<tr>
<th></th>
<th>λcl857 Sam7</th>
<th>λcl857 Sam7</th>
<th>φX174</th>
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</thead>
<tbody>
<tr>
<td>x HindIII</td>
<td>23.13</td>
<td>21.22</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td>9.4</td>
<td>5.15</td>
<td>1.08</td>
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<tr>
<td></td>
<td>6.56</td>
<td>4.97</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>4.36</td>
<td>4.28</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>2.32</td>
<td>3.53</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>2.03</td>
<td>2.03</td>
<td>0.28</td>
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<td></td>
<td>0.98</td>
<td>0.11</td>
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<td>0.83</td>
<td>0.072</td>
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<td></td>
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<td>0.56</td>
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<td></td>
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<td>0.13</td>
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</tbody>
</table>
a rapid boiling method modified from that of Holmes and Quigley (1981; P. T. Barth personal communication, 1987). 20 ml volumes of overnight cultures of plasmid containing strains were pelleted in an MSE Chillspin at 4000 rpm for 7 min, resuspended in 1 ml 1M TES (10 mM Tris-HCl, pH8; 1 mM EDTA; 100 mM NaCl), transferred to an Eppendorf tube and pelleted once more in an MSE microfuge for 1 min. The supernatant was then discarded and the pellet resuspended in 1 ml STET buffer (8% [w/v] sucrose; 50 ml l⁻¹ Triton X100; 0.5 M EDTA; 50mM Tris-HCl, pH 8.0). To this was added 80 µl lysozyme (10 mg ml⁻¹), which was mixed in gently, and the cells lysed by incubation at 37°C for 10 min. The tube was then transferred to a 103°C PEG 400 bath for 90 seconds and centrifuged in an MSE microfuge for 20 min. The supernatant was poured into a clean Eppendorf tube and the DNA precipitated by adding an equal volume of propan-2-ol, mixing and spinning for 15 min in the MSE microfuge. The DNA pellet was freeze-dried and resuspended in 100 µl distilled water.

8.4.2. Restriction analysis.

DNA prepared by the alkaline SDS method was digested with restriction endonucleases in buffers supplied by or recommended by the supplier (Bethesda Research Laboratories, Inc.). Digests were routinely carried out in a volume of 25 µl in the presence of 10 mM spermidine (Bouche, 1981) and 250 µg ml⁻¹ RNaseA, and incubated at 37°C for 1 hour. For agarose gel electrophoresis DNA samples were mixed with one sixth volume loading buffer [2 mg ml⁻¹ HGT agarose [Seakem]; 5 mM Tris-HCl, pH 7.5; 200 mM EDTA; 100 mg ml⁻¹ glycerol; [mixture heated to dissolve]; 0.01 mg ml⁻¹ bromophenol blue; [whole mixture extruded repeatedly through syringe until liquid]]. For separation by polyacrylamide gel electrophoresis samples were mixed with one tenth volume loading buffer (0.25 % bromophenol blue; 0.25 % xylene cyanol;
25% Ficoll (type 400) in H₂O (Maniatis, 1981)).

Restriction fragments were generally separated on HGT Seakem agarose horizontal slab gels of 0.7 - 0.8% (w/v) in TAE buffer (40 mM Tris-acetate, pH 7.4; 1 mM EDTA) and run either overnight at 18 V or for 1.5 - 2 hours at 150 V in TAE buffer containing 5 μg ml⁻¹ ethidium bromide. The fragments were visualized on a short wave-length-UV transilluminator and photographed (Kodak X-Omat, 10-15 seconds exposure, f 4.5). The size of restriction fragments was determined by comparison with λcl327 Sam7 HindIII and λcl327 Sam7 HindIII EcoRI molecular weight markers (Table 8.4.1).

Polyacrylamide gel electrophoresis was used for separation of smaller restriction fragments (less than approximately 1.5 kb). Gels (1 mm thick) were run using a Raven vertical gel kit (IN/96). 30% acrylamide stock solution (290 mg ml⁻¹ acrylamide; 1 mg ml⁻¹ N, N'-methylene bisacylamide) was deionized by treatment with 500 mg ml⁻¹ Amberlite for 30 min, filtered through Whatman 1MM filter paper and stored in the dark at 4°C. 8% plug gels (13.3 ml 30% stock acrylamide in 50 ml; 10 mg ml⁻¹ ammonium persulphate [freshly prepared]; in TBE buffer [89 mM Tris base; 89 mM boric acid; 2.5 mM EDTA; pH 8.3 made as 10x stock solution]) were used with 15% separating gels (50 ml 30% acrylamide stock per 100 ml; 10 mg ml⁻¹ ammonium persulphate; in TBE). TEMED (N,N,N',N'-tetramethylethylenediamide, Eastman Kodak Co., New York) was added (30 μl per 100ml) immediately prior to pouring the gel. Gels were run at 200V for 2.5 hours or overnight at 30V in TBE running buffer. Fragments were visualized by staining for 90 min in 5 mg ml⁻¹ ethidium bromide solution and viewed and photographed as described above.
8.4.3. Cloning procedures.

Vector DNA and the plasmid DNA to be ligated were cut with appropriate restriction enzymes. Where appropriate, fragments were isolated from 0.7 % agarose gels by running onto dialysis membrane inserted into the gel. The membrane was then removed while a 20 V potential was maintained across the gel. DNA was washed from the dialysis membrane with TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) by spinning in a Sarstedt universal tube at 4000 rpm for 1-2 min (MSE centrifuge). All DNA used for ligations was phenol and chloroform extracted and ethanol precipitated as described in section 8.4.1. When necessary isolated DNA was concentrated by adding an equal volume of butan-2-ol, mixing and retaining the bottom (aqueous) phase, repeating as required.

The concentration of DNA was estimated by comparison with a known concentration of λ DNA by running a small sample on an agarose gel. Vector and plasmid DNA were then mixed to give a fragment ratio of 3:1 vector:plasmid DNA such that the vector DNA was at a concentration of 0.1 µg. The ligation reaction was carried out in a total volume of 20 µl containing 18 µl DNA in distilled water, 2 µl 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 16°C overnight, a small sample was run on an agarose gel to check for ligation of the fragments, and the mixture was subsequently used to transform a suitable host strain.
8.4.4. Fill-in of recessed 3' ends of DNA.

Fill-in synthesis was used to remove EcoRI restriction sites from recombinant plasmids. The plasmid was first cut at a unique EcoRI site by restriction endonuclease digestion. The DNA was then phenol/chloroform extracted as described above. 0.5 - 1.0 μg of DNA was then used in a 30 μl reaction containing 3 μl Klenow fill-in buffer (60 mM Tris-HCl, pH 7.5; 60 mM MgCl₂, 10 mM dithiothreitol); 1 μl each of dATP, dCTP, dGTP, dTTP (0.5 mM stock) and 0.5 units Klenow polymerase (BRL). This was incubated for 10 - 15 min at room temperature and the reaction terminated by phenol and chloroform extraction. Finally the plasmid was recircularized by blunt-end ligation using T4 DNA ligase, as described above.

8.5. Strain manipulations.

8.5.1. Transformation.

Transformation was by a modification of the method of Cohen et al. (1972). Overnight cultures of strains were diluted to A₆₀₀ 0.05 and grown through three mass doublings to A₆₀₀ 0.35, with antibiotic selection for any plasmids carried by the strain. Cells were pelleted in an MSE chillspin at 4000 rpm, 0°C for 7 min. The supernatant was poured off and the cells washed by resuspending in 5 ml ice cold 100 mM MgCl₂ and pelleted as before. The cells were then resuspended in ice cold CaCl₂ and incubated on ice for 20 min prior to pelleting as above and resuspending in 1 ml ice cold 100 mM CaCl₂. Apart from KL4330 strains these cells could be kept on ice overnight to increase transformation efficiency. KL4330 strains showed a marked reduction in transformation frequency when treated in this way, and were consequently used immediately for transformation.

250 μl of competent cells were used for each transformation. Approximately 0.25 - 0.5 μg DNA was added to these cells in a sterile
Eppendorf tube and the contents mixed gently. The cells were kept on ice for at least 1 hour, heat shocked at 42°C for 3 min and then placed back on ice for 3 - 10 min. The contents of the tube were then transferred to 5 ml prewarmed Luria broth and plasmid genes expressed for 1 hour (at 37°C) or 1.5 hours (at 30°C) in a shaking water bath. The cells were pelleted once more in an MSE centrifuge and resuspended in 500 μl of phosphate buffer. Dilutions were plated on selective media to give single transformant colonies.

8.5.2. Plating cells for infection with phage λ.

An overnight culture was diluted to A600 0.05 in Luria broth and grown to A600 0.6 - 0.8 with selection for any plasmids carried by the strain. The cells were then pelleted in an MSE centrifuge at 4000 rpm for 8 min, and the supernatant discarded. The pelleted cells were resuspended in 80 % of initial volume in λ-buffer (6 mM Tris-HCl, pH 7.4, 10 mM MgSO₄, 50 μg ml⁻¹ gelatin), and stored at 4°C.

8.5.3. Infection of cells with λ840.

A high titre lysate of λ840 was prepared by mixing 0.1 ml stock phage suspension with 0.1 ml λ plating cells prepared as described above. Tubes were incubated at room temperature for 10 min before adding 3.5 ml molten soft nutrient agar plus 10 mM MgSO₄ to each tube. The mixture was poured onto nutrient agar plates, allowed to set and incubated at 37°C overnight. The soft agar top layer of these plates was then removed with a sterile glass spreader and transferred to sterile 25 ml Sorvall tubes. 1.5 ml of λ buffer was added to each tube and the contents macerated using a sterile spatula. After incubation at 37°C for 30 min the agar was separated by centrifuging for 5 min in a Sorvall RC-5 centrifuge at 8000 rpm, room temperature, in an SS34 Sorvall rotor. The supernatant was transferred to a clean tube and the
phage suspension sterilized by adding one fifth volume chloroform, mixing vigorously and incubating for 20 min at 37°C. The upper aqueous layer was then removed and stored at 4°C.

The phage suspension was titred by adding 0.1 ml of various dilutions to 0.1 ml of λ sensitive cells. After 10 min incubation at room temperature, 3.5 ml soft nutrient agar containing 10mM MgCl₂ was added and poured onto nutrient agar plates. After incubation overnight at 37°C the phage titre could be estimated by counting the individual plaques on these plates.

For introduction of the Tn10del4-HH104 element carried by λ840 into Colibrd⁻1 a BW85 strain harbouring the plasmid was grown from A₆₆₀ 0.05 to A₆₆₀ 0.6, with aeration. MgCl₂ was added to 10mM and 0.4 ml cells were mixed with λ840 stock (1.8 x 10¹⁰ phage ml⁻¹) at a multiplicity of infection of 0.3. After incubation at room temperature for 10 min to allow adsorption the cells were incubated at 37°C for 90 min in a shaking water bath. 0.1 ml aliquots were then plated on Luria agar plates containing 7.5 μg ml⁻¹ tetracycline to select for the presence of the Tn10-specified Tc⁺ marker. Tetracycline resistant colonies were then washed from these plates with 1.0 ml sterile Luria broth, fresh broth was inoculated with 0.1 ml of this suspension and the culture grown overnight at 37°C, with selection. Colibrd⁻1::Tn10 plasmids were transferred to a BW97λ⁺ recipient by conjugation. The mating mixture was 1:10 donors to recipients and mating was carried out at 37°C for 4 hours. The resulting mixture was diluted 1 in 10 and grown up overnight at 37°C. Dilutions were plated onto Luria agar plates to provide colonies to screen for loss of colicin Ib production (see section 8.3.2), which in this case used a λ⁺ colicin sensitive indicator strain.
8.6. Bacterial conjugation.

8.6.1. Mating in liquid culture.

For routine transfer of conjugative plasmids between bacterial strains and for tests of conjugative efficiency, bacteria from overnight cultures were grown for approximately three mass doublings from $A_{600}$ 0.05 to $A_{600}$ 0.35. Cultures were prewarmed at mating temperature for five minutes prior to mixing donor and recipient cells. For tests of conjugative efficiency this ratio was 1:9 donor:recipient (final volume 2.5 ml in a 50 ml sterile flask) and mating was interrupted in a mechanical agitator after 30 min. Routine matings used a 1:1 ratio of donor and recipient cells and were carried out for 1 hour at 37°C or 1.5 hours at 30°C. Mating cultures were shaken gently (Speed 1.5 in a New Brunswick gyrotoratory waterbath), 0.1 ml samples serially diluted and 0.1 ml aliquots plated onto appropriate media to select for recipient cells containing the transferred plasmid.

8.6.2. Mating on solid media.

This method was used to measure the transfer of chromosomal markers from an integrated ColIb oriT site, described in Chapter Four. Donor and recipient cells were grown from $A_{600}$ 0.05 to 0.35. Equal volumes of each (1.0 ml) were then mixed and pipetted onto nitrocellulose disks (Whatmann, 25 mm, 0.45 mm pore size) using a low applied vacuum suction. Filters were placed onto a prewarmed non-selective Luria agar plate, bacteria uppermost. Plates were incubated for 90 min at 30°C. Cells were resuspended by vigorously mixing the filter in 5 ml phosphate buffer. Serial dilutions of these cells were plated onto appropriate selective media, and single colonies obtained counted after incubation at 30°C for 24-48 hours.
8.7. Southern hybridization.

8.7.1. 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) made in TAE buffer (see section 8.4.2) and run in TAE + 0.5 μg ml⁻¹ ethidium bromide. Approximately 3 - 5 μg DNA was run per 5 mm wide slot in a 4.5 mm deep gel such that approximately 0.5 μg DNA was obtained of each fragment required. The desired fragment, visualized using a UV transilluminator, was excised from the gel, excess agarose removed, and the gel slice placed in a preweighed 1.5 ml screw cap 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 ml water to 1 ml gel, and the capped tube placed in boiling water for 7 min. If the fragment was to be labelled immediately the tube was placed in a 37°C water bath. Isolated fragments were stored at -20°C and reboiled for 3 min prior to subsequent labelling reactions.

The labelling reaction, carried out at room temperature, required the following reagents to be added in the order described: distilled water to a total volume 15 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 ^32P-dCTP and 0.6 μl Klenow polymerase (1 unit μl⁻¹, pharmacia).

Oligolabelling buffer is a mixture of solutions A, B, and C (see below) in a ratio 2:5:3, stored at -20°C. Solution A was 1.2 M Tris-HCl, pH 8.0; 120 mM MgCl₂; 1.7 % (v/v) β-mercaptoethanol; 0.5 mM dATP, dGTP and dTTP (Sigma: each 0.1 M triphosphate stock dissolved in 3mM 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 conveniently be run overnight, but
the reaction reaches a plateau after about 3 hours. The reaction was stopped by adding 70 μl stop solution (20 mM NaCl; 20 mM Tris-HCl, pH 7.5; 2 mM EDTA; 0.25 % (w/v) SDS, 1 μM dCTP). The labeled probe was tested for incorporation by placing 1 μl diluted in 10 μl onto a nitrocellulose membrane disk (Whatman; 25 mm, 0.45 μm pore size). After drying, radioactivity on the filter was measured on the 3H-channel of a Packard 3255 liquid scintillation counter. The filter was then washed in ice cold 5 % (w/v) TCA for 2 min and immediately rinsed in distilled water for 3 min, the filter dried and radioactivity of incorporated nucleotides measured as above.

8.7.2. Acid/alkali denaturation of gels.

This procedure was carried out to ensure equal hybridization of small and large DNA fragments (Wahl et al., 1979) and was as described by Southern (1980). The gel was placed in a photographic tray containing 500 ml 0.25 M HCl for 7 min, with gentle agitation, rinsed briefly in distilled water and placed in 500 ml 0.5 M NaOH, 1 M NaCl for 30 min to denature the DNA. The gel was rinsed, as above, and placed in a neutralizing solution (0.5 M Tris-HCl, pH 7.5 in 3 M NaCl) for 30 min. Following rinsing the gel was ready for the blotting procedure.
8.7.3. Blotting procedure.

The method of Southern (1980) was used for the transfer of DNA fragments, except that hybridization was onto a Hybond nylon membrane. The blotting apparatus was constructed by placing a glass platform in a photographic tray flooded with 20 x SSC solution (1 x SSC = 0.15 mM NaCl; 15 mM sodium citrate, pH 7.0). This was covered with two sheets of Whatman 3MM paper soaked in 20 x SSC, which dipped into the tray and acted as a wick. The paper and tray were then covered with cling-film. A hole slightly smaller than the gel was cut in the cling-film to allow liquid to pass through the gel. The gel was placed in position over this hole, and any air bubbles removed. The top surface of the gel was then covered with cling-film such that only those portions which were to be blotted remained exposed. A piece of Hybond nylon membrane (Amersham International) was cut to size and soaked in 3 x SSC, and positioned on the gel, avoiding air bubbles. Surplus liquid was removed from the gel and 1 3MM filter soaked in 3 x SSC and 3 dry 3MM filters were placed on top of the membrane. On top of these was placed a wadd of paper towels, a glass plate and a weight (approximately 1 kg). Wet paper towels were replaced every 15 min for the first 2 hours and transfer was allowed to proceed overnight at room temperature. Following blotting the gel was viewed on a transilluminator to check that transfer of DNA had occurred. The filter was then baked at 80°C for 3 hours, or overnight.

8.7.4. Hybridization of probe sequences to membrane-bound DNA.

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 hour 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 8000. Approximately 0.1
\( \mu g \) denatured oligolabelled probe (see section 8.7.1) was added at this stage and hybridization allowed to proceed overnight at 65\(^\circ\)C with gentle agitation.

Excess labelled DNA was removed by washing repeatedly in 3 x SSC, 0.1 \( % \) (w/v) SDS at 65\(^\circ\)C until the final wash removed no detectable radioactivity. Stringency was determined by the concentration of SSC in the final four washes carried out in SSC, 0.1 \( % \) (w/v) SDS at 65\(^\circ\)C for 15 min each. Filters were blotted dry and covered in cling-film prior to autoradiographing using Kodak X-Omat (1596) X-Ray film.

8.8. Nucleotide sequence determination.

8.8.1. Plasmid sequencing reactions.

Plasmid DNA for sequencing was prepared by a modification of the rapid boiling method of Holmes and Quigley (1981; see section 8.4.1). Approximately 2 \( \mu g \) DNA in 8 \( \mu l \) water was denatured by adding 2 \( \mu l \) 1 M NaOH; 1 mM EDTA, and incubating at room temperature for 5 min. To this was added 3 \( \mu l \) of 3M sodium acetate, 17 \( \mu l \) distilled water and 30 \( \mu l \) propan-2-ol. The mixture was spun in a microfuge for 15 min to pellet the precipitated DNA. The pellet was vacuum desicated for 5 min and the denatured DNA dissolved in 7.5 \( \mu l \) distilled water. To this was added 1.5 \( \mu l \) K-buffer (100 mM Tris-HCl, pH 8.0; 50 mM MgCl\(_2\)) and 1 \( \mu l \) primer oligomer (25-50 \( \mu g \) ml\(^{-1}\); ICI pharmaceuticals division). This was heated to 103\(^\circ\)C in a PEG 400 bath and the primer and plasmid DNA were annealled at 37\(^\circ\)C for 20 min.

For the polymerase reaction 1 \( \mu l \) DNA polymerase I (Klenow fragment, 1 unit ml\(^{-1}\); Boehringer Mannheim), 1 \( \mu l \) SSB (0.6 mg ml\(^{-1}\); Pharmacia) and 3 \( \mu l \) [\( ^{\alpha} \)S] dATP were added and mixed briefly before adding 3 \( \mu l \) of this mixture to the top of four tubes containing 2 \( \mu l \) of dideoxy reaction mixtures. Dideoxy reaction mixtures were: (A) 37.5 \( \mu M \) ddATP, 60 \( \mu M \) dCTP, dGTP and ddTTP; (C) 10 \( \mu M \) ddCTP, 4 \( \mu M \) dCTP, 80 \( \mu M \) ddGTP.
dGTP and dTTP; (G) 25 μM ddGTP, 4 μM dGTP, 80 μM dCTP and dTTP; (T) 125 μM ddTTP, 4 μM dTTP, 80 μM dCTP and dGTP. The tubes were spun briefly in a microfuge to start the reactions which were incubated at 37°C for 15 min.

The chase reactions were started by adding 2 μl chase mix (0.5 mM dATP, dCTP, dGTP, dTTP), and spinning the tubes in a microfuge to mix the contents. Incubation was at 37°C for 10 min. Reactions were stopped by the addition of 4 μl formamide dye mix [1 mg xylene cyanol FF, 1 mg bromophenol blue, 40 μl 0.5M EDTA per 1.0 ml of formamide [formamide was deionized by mixing with 0.5g Amberlite MB1 resin for 30 min, with the resin removed by centrifugation]]. Reaction mixes were heated to 103°C for 1 min prior to polyacrylamide gel electrophoresis (see below).

8.8.2. Denaturing polyacrylamide gel electrophoresis.

Sequencing gels were 6% polyacrylamide, 7M urea and electrophoresis used a BRL vertical gel kit. Glass plates were washed, dried and prepared as follows. The top plate (the plate removed following electrophoresis) was treated with 5-10 ml 2% (v/v) dimethyldichlorosilane which was allowed to dry onto the plate. The plate was then washed with distilled water, dried, washed with ethanol and polished dry with paper towels. The bottom plate (to which the gel was dried) was treated with a mixture of 10 ml ethanol; 1 μl γmethacryloxypropyltrimethoxysilane; 0.3 ml 10% acetic acid (the latter added just before use). This was spread over the plate, which was dried vertically. The plate was then washed with ethanol and polished dry.

Gel plates were separated with plastic spacers (1mm deep) and the two plates taped together with treated sides facing. The separating gel was 6% acrylamide [9 ml 40% acrylamide stock [380 mg ml⁻¹ acrylamide monomer; 20 mg ml⁻¹ bis-acrylamide]]; 6 ml 10 x TBE; 7M urea (25.5g
Analar) to a total volume of 60 ml with distilled water. Immediately prior to pouring, 360 μl 10% ammonium persulphate and 60 μl TEMED were added. Sharks-teeth combs were used to form the wells. Once the gel had set it was preheated to approximately 60°C by running for 30 min at 80 watts.

3 μl samples from sequencing reaction mixes were loaded per lane into the hot gel and run at 80 watts for between 1.5 and 5 hours. The top plate of the gel was carefully removed and the gel fixed in 10% acetic acid for 10 min and washed in distilled water for 10 min to remove the urea. The gel was placed in a fan oven at 70°C until completely dry and then placed in an autoradiograph cassette with X-ray film (Fuji). Autoradiography was generally for 24-48 hours.

8.9. Computer analysis.

Computer programs obtained from the University of Wisconsin Genetics Computer Group (Devereux et al., 1984) were used for the prediction of secondary structure based on the method of Chou and Fasman (1978) and for the generation of hydropathy profiles using the values of Kyte and Doolittle (1982). Programs were run on the Leicester University DEC VAX mainframe.
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ABSTRACT

The Single-Stranded DNA-Binding Protein Gene of Plasmid ColIb-P9.

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The IncII plasmid ColIb-P9 was found to carry a single-stranded DNA-binding protein gene (ssb), and the cloned gene was able to suppress the UV and temperature-sensitivity of an ssb-1 strain of Escherichia coli K-12. Determination of the nucleotide sequence of ColIb ssb demonstrated that the gene shows considerable homology to the ssb gene of plasmid F. In contrast, Southern hybridization techniques indicated that the IncP plasmid RP4 lacks a gene with any extensive homology to F ssb. It was shown that the direction of transfer of ColIb-P9 is such that the ColIb ssb gene, which lies approximately 11 kb from the origin of transfer, is located within the region transferred early during conjugation. The ColIb and F ssb genes are therefore similarly located on their respective plasmids. The ColIb ssb gene was shown to be coordinately expressed with the transfer (tra) genes, suggesting that the ColIb SSB protein may participate in the conjugative process. However, a mutant Colibdrd-1 derivative carrying a Tn903-derived insertion in ssb showed no defect in tests of conjugative efficiency and was apparently maintained stably both following mating and during vegetative growth. Thus no biological role for the ColIb SSB protein was detected. However, unlike the parental plasmid, the ColIb ssb mutants conferred a marked Psi+ (plasmid-mediated SOS inhibition) phenotype on recA441 and recA730 strains. This may result from high level expression of a psi gene due to readthrough from the Tn903 insertion. It is now apparent that many conjugative plasmids previously thought to be unrelated may be derived from a common ancestral plasmid which possessed both ssb and psi genes. It is speculated that the function of the SSB proteins of conjugative plasmids such as ColIb and F may subsequently have been duplicated by analogues derived from newly aquired conjugation systems.