A GENETIC ANALYSIS OF THE TRANSFER GENES OF THE $IncI_1$ PLASMID ColIb-P9.

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ABBRIEVIATIONS

A	Absorbance
Ap	Ampicillin
Ap^R	Ampicillin-resistance
bp	Base pair(s)
cfu	Colony-forming units
Cm	Chloramphenicol
Cm^R	Chloramphenicol-resistance
cpm	Counts per minute
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DDT	Dithiothreitol
dGTP	Deoxyguandine triphosphate
dTTP	Deoxythymidine triphosphate
EDTA	Ethylene diaminetetra-acetic acid
HEPES	N-2-hydroxyethylpiperazine-N-2'-ethane-sulphonic acid
K	1000 revolutions per minute
kb	Kilobase, kilobase pair
Kd	Kilodalton
Km	Kanamycin
Km^R	Kanamycin-resistance
L-agar	Luria agar
moi	multiplicity of infection
Nal	Nalidixic acid
nt	nucleotides
OD	Optical density
PEG	Polyethyleneglycol
pfu	Plaque-forming units
RI	Refractive index
Rif	Rifampicin
SDS	Sodium dodecyl sulphate
SGC	Salts-glucose-casaminoacids medium
Sm	Streptomycin
Sm^R	Streptomycin-resistance
Su^R	Sulphonamide resistance
Tc	Tetracycline
TCA	Trichloroacetic acid
Tc^R	Tetracycline-resistance
Tris	Tris[hydroxymethyl]-aminomethane
Triton-X100	Octylphenoxypolyethoxyethanol
UV	Ultraviolet

CONTENTS

CHAPTER ONE : Introduction.

The aim of this work has been to further our understanding of the process of bacterial conjugation mediated by the $IncI_1$ plasmid ColIb-P9. ColIb is a member of the I incompatibility (Inc) complex which, like the F complex, includes several groups of related plasmids. The plasmids in this complex were originally identified on the basis of their ability to confer sensitivity to I pilus-specific phage, but the group was extended to include the IncB and IncK plasmids on the basis of pilus morphology (Bradley, 1984). Plasmids of the I complex are all incompatible with each other to a greater or lesser extent (Hedges and Datta, 1973; Bird and Pittard, 1982). The plasmids ColIb, R64 and TP110 ($IncI_1$) and R144 ($IncI_1$ + B) are often taken to typify the group (these plasmids of the I incompatibility complex will be referred to as of the IncI group or as I plasmids).

The present models of conjugation have been primarily based on information gained from studies of the F-like plasmids of incompatibility groups IncFI and IncFII. However preliminary studies of the conjugation systems encoded by plasmids belonging to the groups IncI, IncP and IncN have shown that differences at a molecular level exist between these systems and that of the F group plasmids.

The initial observation was made that plasmids from different incompatability groups or complexes shared little DNA homology (Sharp *et al.*, 1973; Ingram, 1973; Falkow *et al.*, 1974; Grindley *et al.*, 1973a) and could not complement F *tra* mutations (Willetts, 1970; Cooke *et al.*, 1970). When more detailed information from other transfer systems became available, differences were also found at a molecular level. For instance the IncI plasmids were found to encode two types of conjugative pilus instead of a single pilus as is the case for F (Bradley, 1983; 1984) and plasmids from other groups were found to encode a range of pili

morphologically and serologically distinct from the thin flexible F pilus (Bradley, 1980a). A conjugative DNA primase activity, not found amongst F-like plasmids, has also been identified for the $IncI_1$ plasmids (Wilkins, 1975) and a similar plasmid encoded activity has been identified for several other conjugative plasmids from a variety of incompatability groups (Lanka and Barth, 1981; see Willetts and Wilkins, 1984). These differences have led to the idea that there may be as many genetically distinct conjugation systems as there are incompatibility groups (Willetts and Wilkins, 1984).

Despite this, there are several features shared by all the conjugation systems so for examined. Firstly they all elaborate sex pili which are involved in establishing cell contact with potential recipient cells (Bradley, 1980a). Secondly they encode a special system for conjugative transfer of a single strand of DNA in to the recipient cell (for a review see Willetts and Wilkins 1984; Boyd and Sherratt, 1986). Thirdly they all possess exclusion systems which act to prevent mating occurring between two cells carrying a related sex factor (Hartskeerl et al., 1983; Willetts and Maule, 1974; Barth, 1979; Winnans and Walker, 1985a). How closely the molecular mechanisms of these analogous processes resemble one another is unknown, but in the following sections I shall use what is known of F plasmid transfer as a general model for conjugation and illustrate where differences have been shown to exist.

1.1 Synthesis of Sex Pili.

An extensive survey by Bradley (1980a; 1980b) has suggested that all conjugative plasmids from gram-negative bacteria encode a sex pilus. He also identified several different morphological forms of pilus and showed that pili from unrelated incompatibility groups were generally serologically distinct. These different sex pili are not thought to be

radically different structures however, and are predicted to share the same general form as the F sex pilus.

The structure of the F pilus was first elucidated by Folkhard et al. (1979) who performed X-ray fiber diffraction studies on purified F pili. They found that the pilin subunits were assembled in to a helical array which formed a hollow cylinder with an outer diameter of 8 nm and an inner diameter of 2 nm. Previous physical studies of F-pilin had shown that the polypeptide subunit was formed into 69% α-helix and 31% random coil (measurements by circular dichroism: Date et al., 1977). They also showed that the pilin tertiary structure was exceptionally resistant to disruption by detergent, urea or heat, suggesting a very compact conformation, possibly expected of a molecule which is exposed to the environment at the cell surface.

Nothing is known of the exact nature of the structure of the other sex pili, but they can be divided in to three groups by morphology; (i) thin flexible pili (~6 nm diameter) which are generally fairly long, (ii) thick flexible plil (~9 nm diameter) and (iii) rigid filaments or rods of various thickness (6 - 11 nm) (Bradley, 1980a; b). By this classification an underlying structure-function relationship was revealed. Plasmids from groups IncN, IncP and IncM, which specify thick rigid pili, have surface obligatory mating systems i.e. conjugation is only efficient on solid surfaces (Dennison and Baumberg, 1975; Bradley et al., 1980). Plasmids with thick flexible pili, such as those from the IncC and IncF groups, can mate in liquid or on solid media and are said to have universal mating systems, although on solid media their efficiency of transfer is increased 45 to 470 times. The plasmids of the I complex transferred equally well in both conditions, and are found to encode both thin flexible and short rigid pili (Bradley, 1984). This general pattern of rigid-surface obligatory, flexible-universal was broken when pili from the IncH13 plasmid, MIP233 were characterized

(Bradley, 1986). These were short pointed rods resembling IncN pili, but the plasmid expressed a universal mating system. These pili were serologically unrelated to all others so far identified, including the other H-complex pili. Blocking conjugative transfer with antiserum raised against MIP233 pili indicated that this was the only surface structure involved in mating.

The mechanism of pilus assembly remains obscure. The activity of at least thirteen tra cistrons is required for elaboration of F pili (Ippen-Ihler, 1985) but the nature of their exact role in this process has only been identified for a few of their gene products. The traA gene, known to encode the F pilin protein (Minkley et al., 1976), has been shown to specify an (Mr) 14,000 d (14 Kd) polypeptide, pro-pilin (Ippen-Ihler et al., 1984). Production of mature pilin protein has been shown to require subsequent processing of this by the traQ product (Moore et al., 1982), thought to be involved in the removal of an unusually long signal sequence of 51 amino acids to produce a 7 Kd polypeptide. Cleavage occurs after a typical signal peptidase cleavage sequence (ala - met - ala_{51}: Frost et al., 1984; Finlay et al., 1984). The rest of the signal is unusual in that it contains a highly charged region as well as the normal hydrophobic core (Ippen-Ihler, 1985). This, taken with the fact that a small quantity of an 8 - 9 Kd traA encoded polypeptide was detected during TraQp mediated processing of the 14 Kd polypeptide, has led to the speculation that TraQp facilitates the removal of the highly charged portion of the signal sequence to allow normal processing of the remaining signal by host cell signal peptidase (Laine et al., 1985).

When the precursor proteins identified from this process were exposed to antiserum raised against mature F pilin, only poor cross-reaction was seen. This suggested that further modifications of the 7 Kd polypeptide must occur before the pilin subunits are assembled into F pili. For instance Woborec et al. (1983) showed that the major antigenic

determinant of mature F pilin was the amino-terminus of the pilin protein. NMR studies have shown that the alanine residue at the amino-terminus is blocked by acetylation (Frost et al., 1984), therefore this modification must occur after the cleavage of the signal sequence and explains why the precursors only gave a poor cross reactivity with mature F pilin-antiserum. It is interesting to note that pilin synthesized by cells which expressed all the tra genes known to be required for pilin biosynthesis (A, L, E, K, B, P, V, W, C, U, N, F, Q and H; see Figure 1.2) except traG, did not produce mature F pilin (Moore et al., 1981a). Ippen-Ihler (1985) reports preliminary results that indicate that addition of traG activity is sufficient to facilitate the necessary acetylation of F pilin. This does not necessarily imply that TraGp itself undertakes the modification. It could be that its presence is required to allow one of the other tra gene products to perform the acetylation.

Further modifications may occur since it has been found that both F-like and EDP208 (IncFV) pili have sugar and phosphate molecules tightly associated with them (Armstrong et al., 1981). (Initially it was thought that these were covalently attached but more careful purification managed to remove most of these moieties.) It is not known whether any of the tra genes are involved in this step but it becomes apparant that the synthesis of mature F pilin is a complex process involving several discrete stages.

Despite this detailed biochemical data concerning the production of mature F pilin, little is known about their assembly into intact pili. The pool of mature pilin protein was found to be located in the inner membrane (Moore et al., 1981b), therefore the subunits must be translocated to the outer membrane, and thence to the exterior of the cell via an assembly mechanism. Evidence from electron microscopy suggests that F pili may be extruded from adhesion zones (Bayer, 1974),

where the inner and outer membranes of the gram-negative bacteria are fused together (Bayer, 1968). Comparisons between F pili and the male specific phage f1 have often been drawn (Brinton, 1971), and it has been observed that the product of gene I of this phage can induce the formation of specialized adhesion zones from which phage particles are released. It is proposed that the other proteins required for phage assembly form a complex in association with the I protein (Lopez and Webster, 1985). (Circumstantial evidence for this role of the I protein comes from their finding that it interacts with host cell thioredoxin, and that this protein is located in the periphery of the cell in an osmotically sensitive compartment, thought to be an adhesion zone.) Biophysical studies of protein secretion have also suggested that insertion of nascent polypeptides in to the outer membrane may induce the formation of adhesion zones (De Leij *et al.*, 1979). Candidates for inducing a pilus assembly adhesion site for F are the *tra* gene products TraGp and 6d, which are found associated with both the inner and outer membrane fractions of the host cell (Achtman *et al.*, 1979; Moore *et al.*, 1981b).

No role has been directly attributed to any other of the other *tra* cistrons involved in pilus assembly or retraction. The study is made more difficult by the interactive nature of these genes, so that a mutation in any one of these cistrons has the affect of abolishing pilus elaboration without providing any information about the molecular processes involved.

1.2 The role of sex pili.

Conjugation is a process involving cell contact. The F pilus has been proposed to make the inital contact between donor and recipient cells, and in mating mixtures cells can be seen connected by these

hair-like filaments (Ou and Anderson, 1970). These authors proposed that the single strand of transferred DNA passed down the hollow interior of the F pilus. However later work by Achtman et al. (1978) demonstrated that F pili per se were not required for DNA transfer, as this could still proceed, after cell contact had been established, in conditions where the pilin subunits were disaggregated. They suggested that the pili mediated the initial contact but the transfer of DNA required a more intimate contact between the cell walls of the mating bacteria. The nature of this contact (often referred to as the conjugation bridge) and how it is achieved is unknown, but the model currently favoured is that pilus retraction, mediated by ordered disaggregation of pilin subunits into the membrane of the donor cell, draws the two cells together (Ippen-Ihler, 1985).

Much indirect evidence of pilus retraction has been gained. By following the infection of F^+ cells by the male-specific phage f1, Jacobsen (1972) found that the average number of F pili decreased when f1 attached to the tips of these pili. Concomitant with this loss, the remaining pili became shorter. Using labelled phage DNA the exact location of the phage genome could be followed, and it was found that after a short lag phage DNA accumulated at the cell surface before entering the cell. It was also shown that no phage DNA was found within the F pilus, again disproving the idea that the pilus is used as a tube to mediate DNA transfer. Studies of the number of pili on the cell surface in various conditions known to affect pilus assembly (Novotny and Fives-Taylor, 1974; 1978; Willetts et al., 1980), have all indicated that changes in the level of pilation are caused by blocking either pilus retraction or elongation. Frost et al. (1984) suggested that naturally occurring variation in the level of pilation of cells with closely related pili may reflect plasmid-specific differences between rates of extrusion and retraction. By labelling donor cell proteins, Sowa et al.

(1983) showed that if pili were constantly removed from the cell surface by shearing, levels of labelled pilin protein in the cell membrane were rapidly depleted, whereas this protein is otherwise stable. The turnover of other outer membrane components was not affected, nor was the pilin depleted in a strain defective in pilus elaboration. In relation to this O'Callaghan et al. (1973) have shown that the restoration of pilus synthesis after arsenate poisoning occurred without de novo protein synthesis, demonstrating that a pool of pilin subunits exists and that pilus elaboration may be ATP dependent. Thus all the evidence points to assembly of F pili from a pool of pilin subunits in the cell membrane and that they are constantly being alternately retracted and extruded. Studies by Burke et al. (1979) and Ou (1973) have indicated that retraction and extrusion of F pili may proceed by different routes, and that retraction induced by cyanide may not be by the same mechanism as that induced by high temperature.

Lawn and Meynell (1972) showed that more thin I pili appeared on the surface of a I^+ cell after treatment with antibody raised against purified I pili, indicating that these pili may also be able to retract and that retraction is blocked by the binding of the immunoglobulin. Data is not currently available concerning the affects of similar treatment on the thick I_1 pilus or on the other types of fexible pili.

1.3 Donor - Recipient Cell Interactions.

The use of male specific phage and Zn^{2+}, which interact specifically with the tips of F pili (Ou and Anderson, 1972; Ou, 1973), has demonstrated that it is the tip that forms the initial mating contact between donor and recipient cells. Purified F pili have also been seen to bind specifically to Escherichia coli cells by their tips (Helmuth and Achtman, 1978). The finding that phage which adhere to the sides of F

pili reduces mating pair formation significantly (Ou, 1973), suggests that either the sides of the pilus also play a role in pair formation or that phage-adsorption to the pilus surface prevents retraction and that this is an important step in formation of mating pairs.

The nature of the receptor on the recipient cell surface is unknown. Several studies were performed to isolate strains with reduced recipient ability , so called Con^- mutants (Achtman et al., 1978b; Havekes et al., 1977a; Ou and Yura, 1982; Reiner, 1974). When the composition of the membranes of these mutants was analysed, it was found that mutants defective in conjugation with F^+ strains ($ConF^-$ mutants) had altered ompA protein or lipopolysaccharide (LPS), whilst $ConI^-$ mutants had normal levels of ompA protein but had an altered LPS composition (Havekes et al., 1978; Achtman et al., 1978b). It was also found that the addition of purified OmpAp and LPS to an F mediated mating, or LPS alone to an $IncI_1$ plasmid-mediated mating inhibited pair formation (Achtman et al., 1978b; Havekes et al., 1977a; 1978), presumably by competing with the pilus for the intact receptor sites on the recipient cells (Sanderson et al., 1981). It was also noted that many $ConF^-$ mutants were still Con^+ in matings with R64 and R100 (IncFII) (Havekes and Hoekstra, 1976; Havekes et al., 1977b; 1978), suggesting that each pilus specified by the different plasmids recognized a separate receptor site on the recipient cell. The finding that most of these Con^- mutations could be overcome in a surface mating system (Achtman et al., 1978b; Havekes et al., 1977b), has led to the idea that the role of the pilus may only be as a physical attachment and this interaction may not generate the mating signal which triggers subsequent events in the conjugation process. The original model that the binding of the tip of the pilus to the recipient cell induced pilus retraction has also been disregarded, since the retraction and elaboration of F pili are now believed to be in constant equilibrium (Willetts et al., 1980; Ippen-Ihler, 1985).

Two stages have been identified in the formation of stable mating pairs. An initial close contact is formed, possibly following pilus retraction, which can be disrupted easily by shear forces and is sensitive to the addition of zinc or male specific phage (Collins and Broda, 1975; Ou, 1973). Following this stage the contact matures to become shear resistant and is also resistant to Zn^{2+} and phage (Ou and Anderson, 1972). It was suggested that the mating signal generated is responsible for the maturation of mating pairs (Ou, 1975). Using different inhibitors of pair formation, Ou and Reim (1976) characterized two stages in the maturation process. Within three minutes of initial contact being made, 50% of mating pairs had become resistant to 1mM Zn^{2+} inhibition, presumably by some step that stabilized the attachment of the pilus tip to the recipient cell. Preincubation of recipient cells with Zn^{2+} however, actually increased recipient ability and so it was concluded that the zinc ions must form part of the receptor site on the female cell (the effect was not seen with Ca^{2+} or Mg^{2+}) and that free zinc probably competes for the attachment site at the pilus tip to inhibit pair formation. Addition of the zinc chelator 1,10-phenanthroline, reduced the frequency of pair formation by 15 - 40%, whilst the efficiency of DNA transfer was reduced 500 fold. This reduction is greater that can be accounted for by the inhibiton of pair formation and implicates Zn^{2+}in a later stage of pair maturation.Within five minutes, the mating pairs had also become resistant to the effects of phenanthroline and allowed DNA transfer to proceed. This characterized the second stage in the stabilization of the mating pair whereby the Zn^{2+} is either no longer required or is no longer accessible to externally added chelator. A third phenomenon has been observed whereby after about 12 minutes the mating pairs become resistant to disruption by the F pilus specific phage MS2. How this relates to the other stages of maturation is unknown, but again indicates that some

conformational change in the interaction between the mating cells has occurred.

Two products of the F tra operon are required to effect pair stabilization. These are traG and traN. TraGp is a large bifunctional protein (116 Kd: Willetts and Maule, 1980), with only the N-terminal region being involved in pilus assembly (Achtman et al., 1972; Manning et al., 1981). Mutants defective in the promoter distal region can form normal pili but are defective in formation of stable mating pairs. The location of TraGp in the inner membrane suggests that it may be part of the pilus assembly complex and that conformation changes generated by a mating signal may be transmitted to the traG product via this complex. Similarly, mutations in traN are defective in stable pair formation (Manning et al., 1981.). The finding that two tra products are involved in this process is not surprising given that the maturation seems to follow a stepwise pathway.

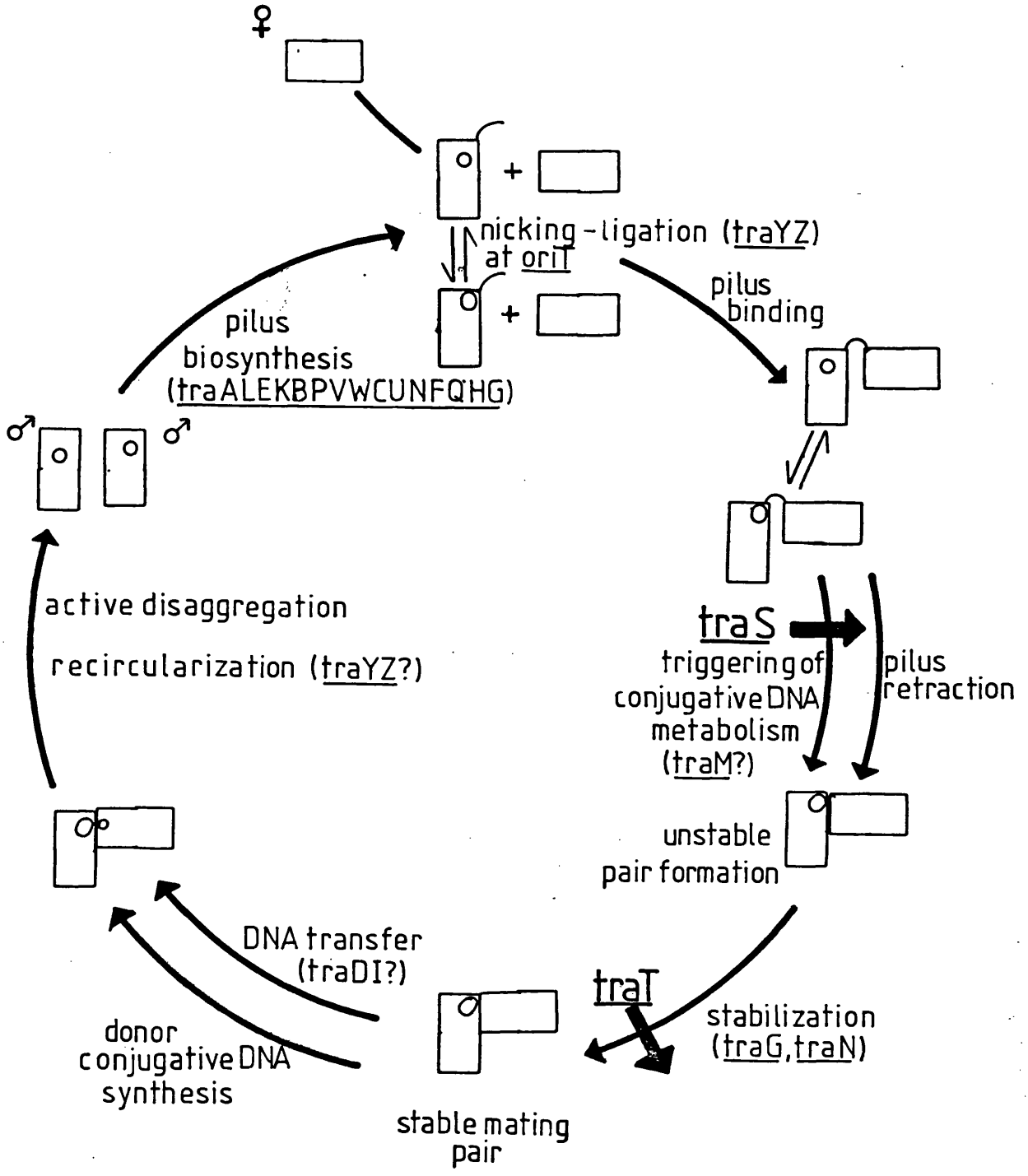
At this point our knowledge of the events leading to the establishment of the conjugation bridge ends. The difficulties involved in transferring the single strand of DNA through two cell membranes has led to the postulate that successful pair formation may be achieved only when an adhesion zone on the recipient cell is brought into apposition with the F induced adhesion zone on the donor cell, and that a localized membrane fusion occurs between the two cells. Whether the retracted F pilus/assembly compex plays any further role in the formation of the bridge is not known. Pair formation however, is independent of the triggering of DNA transfer (Kingsman and Willetts, 1978), and several tra functions not required for pilus assembly are involved in DNA transfer.

1.4 Processes involved in DNA transfer

Although many of the physical processes necessary to transfer a

FIGURE 1.1 : A model for the mating cycle mediated by the F plasmid.

Based on the scheme of Willetts and Skurray (1980; Figure 2). Donor (♂) cells are shown containing the circular plasmid molecule and with an external F pilus. Recipient (♀) cells are represented as an empty cell. Superhelical plasmid DNA is represented as a free circle, whilst nicked circular forms are shown attached to the cell membrane. The F plasmid genes thought to be involved at each stage of the cycle are shown, followed by a query where evidence for their involvement is only circumstantial (see text). Bold arrows which cross the cycle indicate the stage at which traS and traT act to prevent conjugation occurring between two 'male' cells.


♀
+
nicking - ligation (traYZ)
at oriT
+
pilus
binding
pilus
biosynthesis
(traALEKBPVWCUNFQHG)
♂
♂
traS
triggering of
conjugative DNA
metabolism
(traM?)
pilus
retraction
active disaggregation
recircularization (traYZ?)
unstable
pair formation
DNA transfer
(traDI?)
traT
stabilization
(traG, traN)
donor
conjugative DNA
synthesis
stable mating
pair

single strand of plasmid DNA from the donor to recipient cell have been identified, again little is known about how the different steps are achieved. To allow transfer to occur, the double-stranded, covalently closed plasmid DNA molecule must be opened so that the strands can separate. Conjugative transfer is a replicative process, since an identical plasmid molecule is produced in both donor and recipient cells. To achieve this, the transferred strand must be replaced and a complementary strand synthesised in the recipient cell. Finally both molecules are converted into covalently closed supercoiled molecules. These aspects of conjugation have been recently reviewed by Willetts and Wilkins (1984) and a model of the F-plasmid mediated mating cycle was presented by Willetts and Skurray (1980), see Figure 1.1.

Nicking at the origin of tranfer.

Present evidence suggests that conjugative nicking of the F plasmid is achieved by the plasmid encoded traYZ endonuclease (Everett and Willetts, 1980). Despite their close interaction, these two proteins have different cellular locations, TraYp being a membrane protein whilst TraZp is found in the cytoplasm (Manning and Achtman, 1979; Achtman et al., 1979). This suggests that the conjugally replicating plasmid will be located at the boundary of the cytoplasm and inner membrane (possibly at an adhesion zone). Evidence has been found that both the IncFII plasmid R1drd-19 and the $IncI_1$ plasmid R64drd-11 are associated with the cell membrane during conjugal replication (Falkow et al., 1971; Davis and Henry, 1982).

Nicking of a plasmid prior to conjugative transfer is thought to occur at the specific site termed the origin of transfer (oriT). Direct evidence for this was obtained by Everett and Willetts (1980; 1982) who developed an in vitro system for demonstrating nicking at the origin of transfer using the F plasmid oriT site cloned in to a λ bacteriophage.

The sequence of several transfer origins from different plasmids have been determined (R1: Ostermann et al., 1984; F: Thompson et al., 1984; ColE1: Bastia, 1978; RK2: Guiney and Yakobson, 1983) and all have been shown to contain A + T rich regions, which may facilitate the 'melting' of DNA strands and inverted repeats which could form potential stem-loop structures. Work by Mizuuchi et al. (1982) shows that the formation of cruciform structures is favoured by the supercoiling of DNA, so that both strands of a double stranded DNA molecule are formed into a hairpin-loop structure. Therefore covalently closed supercoiled plasmid molecules may exist naturally in the structures predicted for the oriT site from the sequence analysis. Stem-loop structures have often been proposed as recognition sites for proteins which interact with DNA, and the finding by both Thompson et al. (1984) and Bastia (1978) that nick site(s) of plasmids F and ColE1 respectively lay in the loop of one of the possible stem-loop conformations supports the model of the interaction of the nicking enzyme with such a structure.

Initiation of conjugal DNA transfer.

As nicking could be shown to occur in the absence of mating pair formation or conjugative DNA synthesis (Everett and Willetts, 1980), it is assumed that nicking and religation of the single strand at the oriT site is a continuous process, and that strand separation, transfer and replacement synthesis are only initiated when the equilibrium between the two is disturbed by a mating signal. A candidate for setting these processes in motion is the 14 Kd polypeptide product of the traM gene. Plasmids defective in traM fail to trigger conjugative DNA metabolism (Kingsman and Willetts, 1978). The location of this protein within the cell is the inner membrane and it is reported to bind to F plasmid DNA in the region of oriT (Thompson et al., 1984). As the traYZ - oriT nicking complex is also thought to be located at the inner membrane - cytoplasm

interface, a conjugative DNA metabolism complex may be imagined. The final protein thought to be involved in this proposed complex is the traI gene product, helicase I (Abdel-Monem et al., 1983). This DNA-unwinding protein is located in the cytoplasm and mutations in traI also block triggering of conjugal transfer (Kingsman and Willetts, 1978). The unwinding mediated by helicase I is known to unwind the nicked strand in a 5' to 3' direction and F DNA is transferred 5' leading, therefore TraIp must act on the transferred strand (Abdel-Monem et al., 1983). Helicase I requires approximately 200 nt of single-stranded DNA to be able to initiate DNA unwinding. It is proposed that the role of TraMp is to separate the strands following the introduction of a nick into the strand to be transferred by TraYZ endonuclease to open up a region of single stranded DNA on which TraIp can act. In an elegant model proposed by Willetts and Wilkins (1984), the TraIp is anchored at the cytoplasm-membrane interface and the ATP-dependent translocation of helicase I along the DNA as it unwinds it, provides the motive force for DNA transfer.

Evidence in favour of a conjugative DNA metabolism complex comes from studies of the specificities of the proteins thought to interact with the plasmid DNA at the oriT site. For instance, it was shown that the IncF plasmids Flac, R1 and R100 could only mobilize efficiently a recombinant plasmid carrying their own cloned oriT site (Willetts and Wilkins, 1984), and Flac and R64drd-11 plasmids cannot mobilize recombinants carrying the oriT site of the IncP plasmid RK2 (Guiney and Yakobson, 1983). Presumably in each case the triggering proteins fail to recognise plasmid-specific sequences at the origin. Among the F-like plasmids, traY of F, R1 and R100 are not interchangeable whilst only traZ of R1 can complement F traZ mutations (Finlay et al., 1986). Thus it is thought that it is the TraYp moiety of the endonuclease that interacts with the oriT site. Similarly it was found that only those plasmids able to

initiate transfer of the F oriT could complement F traM mutants (Willetts and Maule, 1985). When the oriT sequences of the F-like plasmids were compared, three different sequences were identified which correlated to the three alleles of traM (Willetts and Skurray, 1980). When the sequences of the traM genes from F and R1 were compared, the differences in coding sequence were confined to the N-terminal region. This may represent the region involved in the specific interaction with oriT, while the remainder of the protein has a common functional purpose (Koronakis et al., 1985). An earlier study by Willetts and Maule (1979) demonstrated that the R100 TraIp could not substitute for that of F, but it was suggested that, as this protein is not predicted to interact with origin specific sequences, this specificity may be due to the interaction necessary with other plasmid-specific proteins as part of the DNA transfer complex.

Other evidence for plasmid-specific interactions at the oriT site comes from the isolation of DNA-protein relaxation complexes. In conditions which denature proteins, a nick is introduced into one strand of these superhelical plasmid DNA molecules. Such relaxation complexes were first identified for the mobilizable plasmid ColE1, and were found even in the absence of conjugative plasmids (Blair and Helinski, 1975; Lovett and Helinski, 1975). This indicated that ColE1-encoded mobility functions (mob) were capable of generating the single-stranded DNA nick at the bom site (basis of mobility, required in cis for mobilization), which is known to coincide with the oriT site of ColE1 (Davidson, 1984). This could explain why mobilization of ColE1 is independent of F traYZ, traI and traM activities (Warren and Sherratt, 1977; Everett and Willetts, 1980), as the ColE1-encoded mobility proteins replace the F-specific functions, allowing the non-conjugative plasmid to capitalise on the cell contacts established by conjugative plasmids from different Inc groups (Finnegan and Sherratt, 1982). Such relaxation complexes have

also been identified for the conjugative plasmid RK2 (Guiney and Helinski, 1979). They found that nicking occurred at the relaxation site (rlx) and that the oriT site of this plasmid mapped in the same region as rlx. Furthermore, the rlx sequence inserted in to pBR322 promoted the mobilization of this plasmid by RK2 derivatives, but not by Flac plasmids, suggesting that only RK2 specified proteins could interact with the RK2 origin of transfer. Similarly it was found amongst IncI plasmids, that a recombinant plasmid carrying the ColIb oriT site was only efficiently mobilized by plamids which encoded the I_1 conjugation system (Wilkins et al., 1985). These data all support the model of a specific protein-oriT interaction required to initiate the transfer of DNA.

The mating signal.

The nature of the mating signal that triggers these interactions is not known. What is known is that stabilization of mating pairs is not involved, since conjugative DNA synthesis can be induced in both traG and traN mutants where stabilization is blocked (Kingsman and Willetts, 1978). De novo protein synthesis is not involved since all the F transfer genes are coordinately expressed under the control of traJ (Achtman et al., 1971; 1972; Achtman, 1973; Willetts, 1977; Finnegan and Willetts, 1973), and pretreatment of donor cells with rifampin did not prevent transfer of F plasmid DNA (Kingsman and Willetts, 1978) nor the mobilization of ColE1 (Willetts and Wilkins, 1984.). Therefore mating pair formation may induce an allosteric change in the structure of a triggering protein (this might be TraMp).

Ou and Yura (1982) have indicated that components of the recipient cell may be involved in generating the mating signal. They identified a $ConF^-$ mutant that allowed mating-aggregate formation but formed transconjugants with low frequencies. These had lost an 82 Kd outer

membrane protein and failed to induce conjugal DNA replication in the donor cell.

DNA transfer.

Specific mechanisms involved in the transfer of the single strand of DNA remain unclear, but the TraDp (78 Kd) may play a central role in this process and may function to link together the DNA metabolism complex and the pilus assembly complex. Kingsman and Willetts (1978) initially demonstrated that traD mutants failed to transfer plasmid DNA. They also found that these mutations caused a five fold reduction in the rate of conjugative DNA synthesis in the donor cell, although this did not account for its effect on DNA transfer since conjugative DNA synthesis is not required for strand transfer to proceed. Later it was shown that missense mutations in traD caused two to three times the number of pili to be produced per cell compared to the number found on a wild type F^+ cell, but the affect of traD on DNA transfer was shown to act at a stage after extracellular pili were required (Panicker and Minkley, 1985). They also reported current work which indicates that TraDp may serve as the membrane anchor for DNA helicase I (TraIp). As it affects so many parts of conjugation, TraDp may form a central part in the membrane-transfer complex. A 78 Kd protein corresponding to the traD gene product has been located to both inner and outer membranes (Achtman et al., 1979.), and it has been suggested that this protein may span the adhesion zone and play a central role in conduction of the DNA in conjunction with other conjugation proteins in the complex.

Components of the recipient cell membrane have been implicated in DNA transfer by the finding that some $ConF^-$ mutants can induce conjugative DNA synthesis in the donor cell, but fail to form transconjugants. In these strains alterations in both the inner and outer membrane protein profiles were seen (Ou and Yura, 1982.) suggesting that recipient cell

components are also required to allow DNA transfer.

It has been demonstrated for plasmids of the IncFI, IncFII and $IncI_1$ groups, and for ColE1, that a specific strand of plasmid DNA is transferred into the recipient cell (Vapnek and Rupp, 1970; 1971; Vapnek et al., 1971; Boyd and Sherratt, 1986). The transfer of F is unidirectional, as it is for the IncP plasmids RP4 (Grinter, 1981; Al-Doori et al., 1982) and for ColE1 (Boyd and Sherratt, 1986), and the gradient of transmission of chromosomal markers by an integrated IncI plasmid , R144, indicates that this is also transferred unidirectionally (Datta and Barth, 1976). The polarity of transfer has only been determined for the plasmids F and ColE1 (Ohki and Tomizawa, 1968; Ihler and Rupp, 1969; Boyd and Sherratt, 1968) which are transferred with the 5' terminus leading. Transfer of F and RP4 is initiated at oriT and proceeds such that the coding region of the conjugation genes is transferred last.

It has recently been shown that F encodes a single-stranded DNA binding protein (ssf) that maps near the oriT site in the region of DNA which is transferred first in conjugation (the leader region) (Golub and Low, 1986a; Kolodkin et al., 1983). It was also found that homology existed between the F leader region and the plasmids from Inc groups K, B, I_1 and Y (Golub and Low, 1986a). The ssb gene (single-stranded DNA binding protein) of the $IncI_1$ plasmid ColIb-P9 has recently been found to lie near the oriT site (C. Howland, unpublished data), in a position such that if this gene was located in the leader region of ColIb, known tra genes of the plasmid would be transferred after the rest of the plasmid (see Figure 1.4). The leader region of F is known to express four polypeptides (Ray and Skurray, 1983; Cram et al., 1984) including the ssf gene product. Whether the homology within this region of plasmids from different incompatibility groups implies a functional relationship for the proteins encoded therein remains a matter of

speculation. A role for the plasmid-encoded single-stranded binding protein in the recipient cell could be envisaged, but a recent report from Golub and Low (1986b) shows that ssf mutations have no effect on conjugative DNA transfer in E coli, despite the evidence that this gene is coordinately expressed with the transfer genes.

Conjugative DNA synthesis.

Much work has been undertaken on this subject, with most data being obtained from studies of F and the $IncI_1$ plamids ColIbdrd-1 and R64drd-11 (for a review see Willetts and Wilkins, 1984). Whilst both these groups of plasmids are thought to use host cell DNA polymerase III holoenzyme to achieve elongation of the nascent DNA in both donor and recipient cells (Wilkins and Hollom, 1974; Kingsman and Willetts, 1979), a major difference has been found in how RNA primers are generated to initiate DNA elongation.

Conjugative DNA synthesis requires the formation of a 3'-OH primer terminus. In F, priming of complementary strand DNA synthesis in the recipient cell is thought to be achieved by either RNA polymerase or the primosome, since the process was rifampin-resistant in dna^+ cells but was rifampin-sensitive in dnaB cells (see Willetts and Wilkins, 1984). In contrast to this, complementary strand synthesis of $IncI_1$ plasmids does not require activity of either RNA polymerase, dnaG primase or the primosome in the recipient cell (Boulnois and Wilkins, 1978; 1979; Wilkins and Hollom, 1974). These plasmids specify their own primase activity encoded in the sog gene (Lanka et al., 1979; Boulnois and Wilkins, 1979) and it has been shown that this mediates priming of conjugal DNA synthesis in the donor and recipient cells (Chatfield et al., 1982; see Section 1.7). The IncP plasmid, RP4, has been shown to encode an analogous but genetically distinct primase activity (Lanka and Barth, 1981), and evidence suggests that this too is transmitted in an

active form to the recipient cell to prime conjugative DNA synthesis (Merryweather et al., 1986a). This finding underlines the fact that not all models of conjugation derived from the study of F plasmid transfer can be extrapolated to plasmids of the other incompatibility groups.

Recircularisation of the transferred strand.

The final step to be achieved in the transfer process is the recircularisation of the plasmid DNA in the recipient cell (for a review see Willetts and Wilkins, 1984). The model currently favoured is that after nicking at the origin of transfer, the traYZ product remains covalently attached to the 5' end of the DNA, conserving the energy of the phosphodiester bond. This was proposed after it was found that ColE1 and ColE2 DNA isolated from relaxation complexes were not suitable substrates for T4 ligase, and the 60 Kd relaxation complex-protein was covalently attached to the 5' end of the DNA molecule (Guiney and Helinski, 1975; Warren et al., 1978). Thompson et al. (1984) showed that nicks introduced into the cloned F oriT site were also unable to be ligated by T4 ligase, suggesting that the ends of the nick were also blocked. Assuming that this blockage is the traYZ endonuclease, it is thought that this complex can recognise the two halves of the cleaved oriT site and can religate them using the energy of its own release. This would be analogous to the function of the ϕX174 cistron A protein which, after nicking the phage DNA remains bound to the 5' end of the molecule and then mediates the religation (Eisenberg et al., 1977). If the site of DNA transfer is a fusion of two adhesion zones, the traYZ endonuclease-DNA complex need not be physically transferred into the recipient cell but may remain located at the fusion site with the plasmid DNA attached. It must be noted that after transfer of a monomeric unit of DNA has been completed, active disaggregation occurs (Achtman et al., 1978a). A mechanism could be envisaged whereby release of the plasmid

DNA from the traYZ protein may trigger conformational changes in the transfer complex which initiates the disaggregation process.

1.5 Surface Exclusion.

The expression of all the proteins required for a complex conjugation system places a heavy metabolic load on a cell. Some evidence also suggests that elaboration of sex pili make F^+ and I^+ cells more susceptible to detergents and other lytic agents (Salisbury et al., 1972; Dowman and Meynell, 1970) and also confers sensitivity to various male-specific phage on its host. Therefore the natural state of most transfer genes is repressed, although exceptions to this rule exist (notably F and RP4: Willetts and Skurray, 1980; Bradley, 1980a). Bradley (1984) describes a scenario where, in a population of cells carrying repressed plasmids, a few cells would express the conjugation genes at a low level. When suitable recipient cells become available, these donor cells would complete successful transfer of plasmid DNA. It is known that the repression of the $IncI_1$ transfer genes in a newly infected cell can take two to seven generations to become established (Monk and Clowes, 1964) and therefore these newly infected donors can mediate the rapid spread of the sex factor through a population (see Lunquist and Levin, 1986). However, if the cells carrying the sex factor could not distinguish between cells carrying the same plasmid and a suitable non-infected cell, such a repression system would not be possible, to the detriment of the host cell. Thus it is thought the exclusion systems have evolved which allow plasmid carrying cells to identify like cells. Obviously exclusion also confers metabolic economy on the system as it does not waste energy allowing the plasmid to invade a bacterium which it already occupies.

Many exclusion groups have been identified, and only the donor cells

carrying plasmids of the same group will be effectively inhibited from transferring DNA. Amongst F-like plasmids a correlation was found between the exclusion group and the pilus variant, suggesting an interaction of the tip of the pilus and the exclusion system (Willetts and Maule, 1974). This was supported by the finding that the mixed pili expressed from the donor cells carrying two related F-like plasmids did not recognise either of the parental exclusion systems (Willetts and Maule, 1974; 1985). The genes and gene products involved in the exclusion systems of F and R144 have been well characterised and although they achieve the same effect, are apparently radically different.

F exclusion.

The F plasmid exclusion system involves the expression of two transfer genes, traS and traT (Achtman et al., 1980). The product of traS gene (TraSp) is an 18 Kd inner membrane protein and TraTp is a 25 Kd lipoprotein located in the outer membrane (Achtman et al., 1977: Minkley and Ippen-Ihler, 1977; Perumal and Minkley, 1984). Achtman et al. (1977) found that mutations in either one of these genes only partially abolished exclusion, indicating that the two genes had an additive effect on the exclusion index. Mutations in traT did not effect initial pair formation, but inhibited their maturation into stable mating pairs (Achtman et al., 1977; Minkley and Willetts, 1984) and are thought to act by blocking the interaction of the F pilus with a receptor on the cell surface (this proposed function correlates with the finding that TraTp spans the outer membrane and is exposed on the outer surface: Perumal and Minkley, 1984). Many proteins which have a final location in the outer membrane have amino-terminal signal sequences which facilitates their transport through the lipid bilayer (see review by Randall and Hardy, 1984.). Most of the F transfer proteins, with the exception of the pilus protein, are thought to be exported without proteolytic processing

(Achtman et al., 1979) and it has been suggested that the assembly of the transfer complex allows them to become correctly located. TraTp however, is found to have a signal sequence which may imply that it has a location independent of the other tra gene products (Minkley, 1984; Perumal and Minkley, 1984). TraTp is found in approximately 20,000 copies per cell (Minkley and Willetts, 1984) and is thought to be expressed at low levels even when the other tra genes are repressed despite a failure to find a traJ-independent promoter sequence such as that identified for R100 traT gene (Rashtchian et al., 1983; Finlay and Paranchych, 1986). It is thought that TraTp has to interact with all the possible receptor sites (which may be adhesion zones) on the cell surface to be able to block the mating process. Another observed effect of TraTp is to confer serum resistance on cells. Ogata et al. (1982) and Moll et al. (1980) identified the traT genes of R100 and R65 as causing resistance to killing by complement. As adhesion zones have been proposed as the site of action of complement, this may be a coincidental property of the presence of TraTp at these sites whilst involved in blocking them as recepter sites for pili. Sequencing of the traT genes of F and R100, which belong to different exclusion groups, has identified considerable homology between the two gene products (only one amino acid residue was altered) and the proteins are immunologically identical. However this small change must be sufficient to provide the plasmid specificity of the traT products, as illustrated by the different inhibitory effects of purified TraTp on F and R100 mediated matings (Minkley and Willetts, 1984).

Mutations in traS reduced recipient ability ~1000 fold, despite the fact that normal mating aggregation was seen. It had already been shown that entry exclusion acted to prevent DNA entering the recipient cell (Sheehy et al., 1972) and these mutations had no effect on pair formation, but it was found that TraSp inhibited triggering of conjugal

DNA metabolism (Achtman et al., 1977). Its location in the inner membrane may block later stages in generation of the mating signal. This protein is only produced in small amounts and is under control of traJ. It may be that TraSp modifies the transfer complex in some way, rather than acting as a non-specific block.

Other exclusion systems.

The exclusion system of R144 seems to be analogous to a traS type of mechanism. Hartskeerl et al. (1983; 1985a) cloned the R144 entry exclusion gene (exc) and showed that this gene also causes exclusion of ColIb and R64 but not of IncF plasmids. Expression of the exc gene had no effect on cell aggregation, and the level of exclusion (normally ~100 fold) increased in a surface mating system, indicating that the exclusion was acting at a stage beyond mating aggregate formation (Hartskeerl and Hoekstra, 1984). Two proteins with overlapping coding sequences were identified from the cloned exc determinant, of 13 Kd and 19 Kd. The 19 Kd protein was found to be bound to the periplasmic side of the inner membrane and also in significant amounts in a soluble form in the cytoplasm. Presence of the 19 Kd polypeptide was shown to be essential for entry exclusion but involvement of the 13 Kd protein seemed unlikely but could not be ruled out (Hartskeerl et al., 1985b). The exclusion determinant of ColIb (eex) has also been cloned and will be discussed further in section 1.7.

An entry exclusion system has also been identified for the IncP plasmid RP4 (Barth, 1979; Barth et al., 1978) and it has been shown to involve at least two genes which, like F traS and traT, have an additive effect on the exclusion index. Winans and Walker (1985a) report the finding of a single region required for surface exclusion for the IncN plasmid pKM101. In both cases these genes have been found to lie within DNA containing other conjugation genes but (with the possible exception

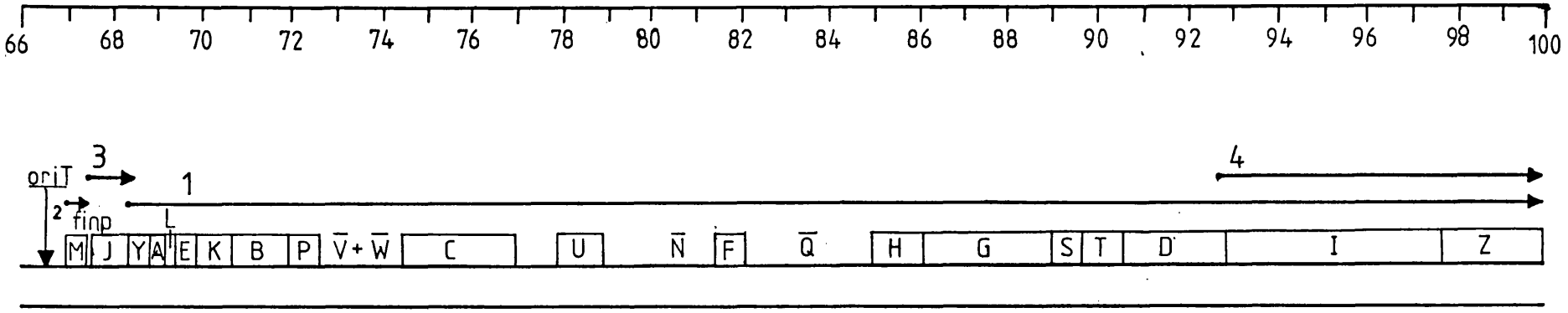
of RP4 : Barth, 1979) are not thought to be involved in the transfer process. The exclusion system pKM101 is expressed independently of the tra repression system (Winans and Walker, 1985a).

1.6 Genetic organisation of transfer regions.

The elucidation of the genetic organisation of the F plasmid transfer genes was the result of extensive research by several groups. Much laborious work was done to isolate mutants that were transfer deficient, or were resistant to male specific phage. Classical complementation analyses were then performed to identify the tra cistrons, using either related but compatible IncFII plasmids or by forming transient heterozygotes. Later the use of isolated genes from the tra region cloned in to λ phage provided an easier way to perform the complementation tests, and the polar effects of transposons and phage Mu lead to an understanding of the transcriptional organisation of the tra cistrons (for a review, see Willetts and Skurray, 1980).

Despite nearly 16 years work by numerous groups, the location of the genes of the F tra operon has not been fully elucidated, and the precise function of only a few has been determined. The genetic map of the F tra region is summarised in Figure 1.2. The salient features of this are the organisation of most of the tra genes into one long traYZ operon (Helmuth and Achtman, 1975) and the location of the origin of transfer at one end of the tra region. Outside the traYZ operon are the traM and traJ cistrons and the fertility inhibition gene finP. Transcription from both the traM and traYZ promoters is dependent on the product of traJ (Gaffney et al., 1983), and in F the traJ gene (and thereby the transfer genes) is constitutively expressed. A traJ-independent promoter exists upsteam of the traI gene allowing expression of traI and traZ at low levels even when TraJp is not produced (Gaffney et al., 1983).

FIGURE 1.2 : Map of the F transfer genes.



Numbers above the map indicate kilobase coordinates on F. Lettered tra genes are shown in boxes, indicating the extent of coding region where known (based on Figure 1; Ippen Ihler, 1985). The exact positions of genes in the traP - traH region are uncertain, and additional genes may be present. The location of the finP gene and oriT site are also shown.
The arrows above the map show: (1) and (2) TraJp-stimulated transcripts; (3) the finOP-regulated traJ transcript; (4) a low level TraJp-independent transcript for traI and traZ (see text).

As was described in Section 1.1 at least 13 tra cistrons are required for the expression of F pili (A, L, E, K, B, V, W, C, U, F, Q, H, and G). These are arranged in a block together, only interrupted by traN (triggering) and traP (no known function). Gaps still exist in the central portion of the map and the detection of gene products from this region more numerous than known cistrons suggests that extra genes may also be involved in pilus synthesis (Ippen Ihler et al., 1984). The products of tra genes M, Y, D, I, and Z are involved in DNA transfer and TraGp and TraNp are responsible for stabilization of mating pairs (see sections 1.3 and 1.4 respectively). The genes required for exclusion, traS and traT, are found together in the middle of the transfer operon but have no known role in DNA transfer itself (see Section 1.5).

Although the F plasmid was isolated as a naturally derepressed (drd) conjugative plasmid, the transfer genes of most of the other F-like plasmids are normally repressed. Repression of these plasmids is mediated by the products of the finO and finP genes, which together form the FinOP inhibitor of transcription from traJ. In the absence of traJ, the expression of all the transfer genes is abolished (Finnegan and Willetts, 1973; Willetts, 1977b). When supplied in trans, the finO gene of R100 was found to repress F traJ expression, suggesting that this plasmid had a normal finP gene but was defective in finO (Finnegan and Willetts, 1973; Grindley et al., 1973b), possibly due to the insertion of an IS30 sequence into the gene (Cheah and Skurray, pers. comm, in Willetts and Maule, 1985). Complementation studies involving several of the F-like plasmids have revealed that the finO gene product is fairly plasmid non-specific (only two alleles of finO were found), but six alleles of finP were identified which corresponded to the plasmid specificities of the traJ gene product in each case (Willetts and Maule, 1985). This correlation may result from the fact that these two genes overlap, the finP gene product thought to be a small RNA, complementary

to the traJ mRNA (Fee and Dempsey, 1986). The FinOP inhibitor prevents transcription from traJ (Willetts, 1977; Gaffney et al., 1983) and Fee and Dempsey suggest that a likely site of interaction of the finP RNA is the RNA polymerase binding site upstream of the traJ gene, which lies within the finP coding region. The product of the finO gene has been identified as a 22 Kd polypeptide (Timmis et al., 1978) and may serve to stabilize the interaction between the finP RNA and the traJ leader region. In accordance with this model is the finding that, despite considerable divergence between F and R1 plasmids in this region, the proposed operator site in each case where the plasmid non-specific finO protein would bind, is highly conserved (Finlay et al., 1986).

Some ambiguities still exist about the role of the traJ protein. TraJp has been identified as a 24 Kd polypeptide. When overproduced from an Flac::λ traJ lysogen, it was found to be located in the outer membrane of the host cell (Cuozzo et al., 1984). This agrees with the findings of Achtman et al. (1979) and like the other tra proteins, TraJp is transported to the outer membrane without proteolytic cleavage of a signal sequence. It is difficult to reconcile this location in the outer membrane with its role in transcriptional regulation of the tra genes, and yet Cuozzo et al. (1984) have shown that localisation of TraJp in the cell envelope, or at least its translocation to an envelope site, is required for tra operon transcription (see also Sambucetti et al., 1982). Whether TraJp exerts its control on the other tra genes directly or indirectly remains unclear. It has been suggested that the traJ product, possibly in an oligomeric form, may span the membrane and acts at the cytoplasm interface on the plasmid DNA. Some clues to its mode of action may come from studies of chromosomal mutations that cause premature termination of transcription of both traJ and the traYZ operon, suggesting that it may modify cellular mechanisms to allow expression of plasmid-encoded genes, but no conclusive data has yet been produced

(Beutin and Achtman, 1979; Beutin et al., 1981; Sambucetta et al., 1983; Rehemtulla et al., 1986).

The transfer region of RP4 (IncP).

Restriction data from the other well studied plasmids of the IncP group suggest that they are very similar, if not identical and therefore RP4 can be taken as the archetypal IncP plasmid (RP1: Grinsted et al., 1977; RP4: Barth and Grinter, 1977; R68: Holloway, 1979; RK2: Figurski et al., 1976).

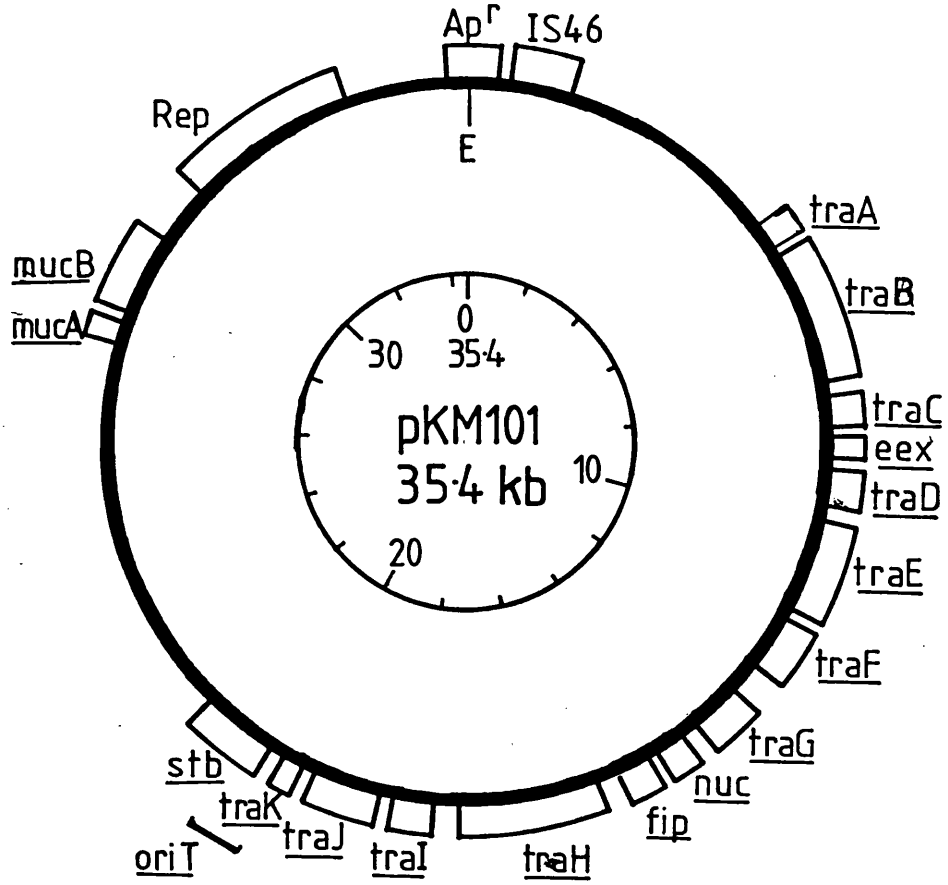
The organisation of the transfer genes of RP4 was investigated by Barth et al. (1978) using the transposon Tn*7*. They identified three blocks of *tra* genes, Tra1 and Tra2 being separated by the kanamycin resistance determinant. The *tra* region of the closely related plasmid RK2 consists of one continous segment of DNA (Figurski et al., 1976) and the Km^R determinant of RP4 was probably acquired from a transposable element, interrupting a previously contiguous region. The origin of transfer was located at one end of Tra1. This block of *tra* genes also encompasses the *pri* gene (primase gene: Lanka and Barth, 1981; Lanka et al., 1984). The primase gene covers 3.2 kb and is the penultimate gene in a trascriptional unit containing four genes. It is transcribed towards the Km^R determinant, as shown in Figure 1.3. Two surface exclusion determinants have been located, one in each of Tra2 and Tra3 (Barth, 1979).

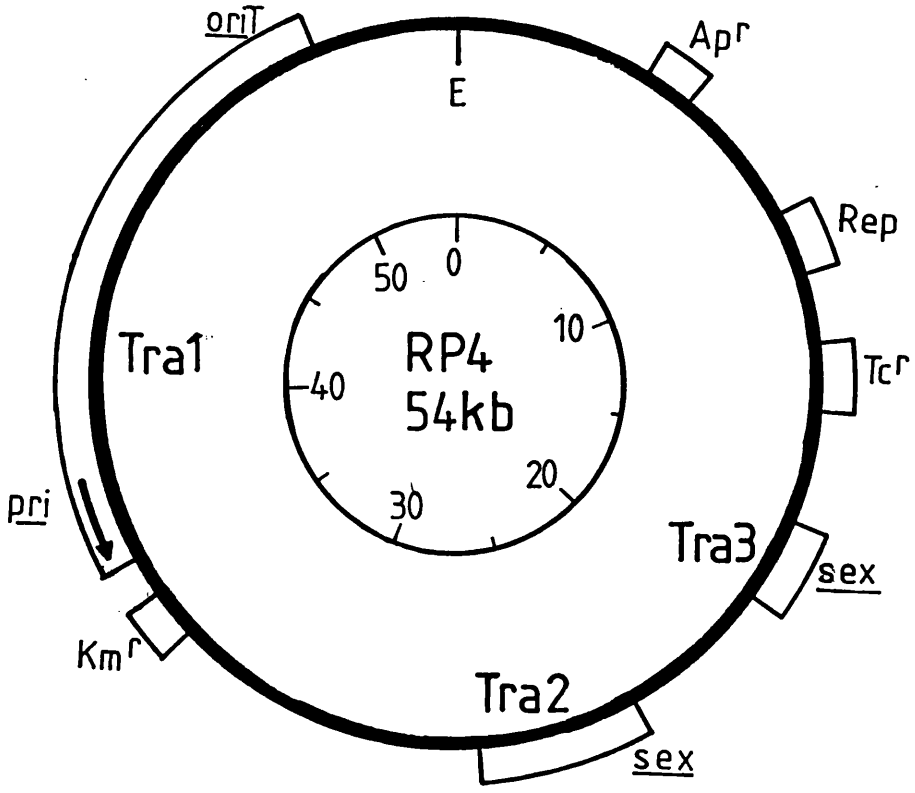
Plasmids of the IncP group specify P-1 type pili (Bradley, 1980a) which morphologically resemble the IncN pilus, being thick rigid structures with pointed distal tips. Several Tn*7* mutants in the Tra1 region affected pilus synthesis, indicating that this region contains pilus assembly genes. When IncP male-specific phage attach to the sides of the P pilus, an increase in the number of pili per cell was seen, which was taken as an indication that these pili are retractile, becoming

FIGURE 1.3 : The transfer regions of pKM101 (IncN) and RP4 (IncP).

pKM101 : Diagram taken from Winans and Walker (1985b). The complementation groups traA through to traK and the oriT were identified by Tn5 mutagenesis. Also identified by this method is the entry exclusion determinant (eex). Amongst these tra genes is found the nuclease gene (nuc) (Winans and Walker, 1983) and the fip determinant which is involved in the inhibition of the fertility of IncP plasmids in the same donor cell as the IncN plasmid (Winans and Walker, 1985c). On the opposite side of the plasmid are found the Rep region, essential for replication, the mutagenesis enhancement determinants (muc) (Perry and Walker, 1982) and the ampicillin-resistance determinant (Ap^R).

RP4 : Map based on those of Barth (1979) and Merryweather (1986). Three blocks of transfer genes have been identified by Tn7 mutagenesis (Tra1, Tra2 and Tra3). Tra1 is thought to contain the genes required for the synthesis of the IncP pilus and also encodes the plasmid primase (pri : arrow indicates the direction of transcription and extent of the coding region of this gene). At the other end of Tra1 is found the oriT site. The entry exclusion determinants (sex) have been located in Tra2 and Tra3. These two blocks of transfer genes are separated from Tra1 by the kanamycin-resistance determinant (Km^R). Also shown, on the opposite side of the plasmid, are the ampicillin- and tetracycline-resistance determinants (Ap^R and Tc^R) and the Rep region.


Ap^r
IS46
E
Rep
mucB
mucA
traA
traB
traC
eex
traD
traE
traF
traG
nuc
fip
traH
traI
traJ
traK
stb
oriT
0
30
35·4
10
20
pKM101
35·4 kb


oriT
E
Ap^r
Rep
Tc^r
Tra3
sex
Tra2
sex
Km^r
pri
Tra1
0
10
20
30
40
50
RP4
54kb

locked in the extended position by the adsorption of the phage.

The IncN conjugation system.

The organisation of the tra genes of two IncN plasmids has been studied; pCU1 (Thatte et al., 1985) and pKM101 (Winans and Walker, 1985b). The tra genes of both of these plasmids have been localised to a contiguous segment of DNA, although Tn5 insertional analysis has suggested that transcription is discontinuous in both cases, unlike the long traY-Z operon found for F. The IncN group plasmids encode short rigid pili (9.5 nm diameter) which are brittle and easily detached from the cell surface. These pili are pointed at the distal tip, and male-specific phage were seen to adhere to the tip of the pilus (Bradley, 1979). The protruding length of the N pilus from the cell surface was measured as 0.1 to 0.5 μm, although free pili appeared to be longer. Following infection by male specific phage, the average pilus length was seen to shorten and phage were were found to accumulate at the cell surface. Again this has been taken as an indication that pilus retraction can occur. Dennison and Baumberg (1975) reported the rescue of IncN transfer frequencies on solid media, agreeing with the reported properties of thick rigid pili (Bradley, 1980b).

Winans and Walker (1985b) identified three TRA regions for pKM101, each of 3 - 4 kb, which accounted for ~40% of the total genome (~32 kb: Langer et al., 1985), therefore the total region required to express the transfer system of pKM101 (~13 kb) is considerably less than is required for F (~30 kb). All of the DNA in the first two regions was required for sensitivity to IncN male-specific phage (i.e. pilus expression) and oriT was found to be located in 1.2 kb of DNA at one end of the tra region (see Figure 1.3). Using Tn5 mutagenesis Winans and Walker identified eleven complementation groups which, assuming Tn5 is polar in this system, probably represent independent transcriptional units. The

function of those genes affected remains obscure. The single entry exclusion determinant of pKM101 was located in a 0.7 kb fragment which lies between TRAI and TRAII. This gene was not coordinately expressed with the other transfer genes, and Tn*5* mutagenesis showed that efficient exclusion is not required for transfer (Winans and Walker, 1985a).

A plasmid-encoded endonuclease (*nuc*) has also been located in a 0.8 kb region which lies between TRAII and TRAIII. Again this gene was not coordinately expressed with the transfer genes and a Tn*5* insertion that inactivated the *nuc* gene was found to be Tra^+ (Winans and Walker, 1983). The enzyme has been extensively characterised (Lackey *et al.*, 1977) and the gene product was identified as a 16 Kd polypeptide, which required a divalent cation for activity (optimum activity was achieved with 10mM Mg^{2+}, but this could be substituted by Ca^{2+}, Zn^{2+} or Co^{2+}). It acted on single-stranded and double-stranded DNA equally well, with no apparant base specificity, forming 5' phosphate ends in an ATP-independent reaction. The pKM101-encoded nuclease differs from other host-encoded endonucleases in that it is EDTA resistant. In all other aspects (location, expression and function) it seemed to resemble the host-encoded endonuclease I (Winans and Walker, 1983). Many other conjugative plasmids were tested for expression of a similar endonuclease and it was found that plasmids from any one group all had the same phenotype. Surprisingly of those plasmids tested, only the plasmids of the I complex scored positively for nuclease expression, whilst the IncP and IncW plasmids, considered to be more closely related to the Inc N plasmids, did not encode an EDTA-resistant nuclease.

Mutations in the *nuc* gene also indicated that it has no role in plasmid stimulated DNA repair and mutagenesis (mediated by the *muc* genes), entry exclusion or fertility inhibition of P group plasmids mediated by pKM101 (Winans and Walker, 1983).

The *tra* region of another IncN plasmid, pCU1, has been investigated

using Tn5 mutagenesis (Thatte et al., 1985), and it was found that the conjugation genes of this plasmid were also contained in a single contiguous segment of the plasmid, containing at least eight complementation groups (presumed to represent transcriptional units), six of which were required for production of the N pilus. No transposon insertions were obtained for two small stretches of DNA within the tra region and when the physical map of pCU1 and pKM101 were aligned, these regions were found to correspond to the sites of the nuclease and entry exclusion determinants of pKM101, suggesting that the tra regions of the two plasmids are closely related. Again the cis-acting site oriT was found to fall at the end of the tra genes of both pCU1 and pKM101.

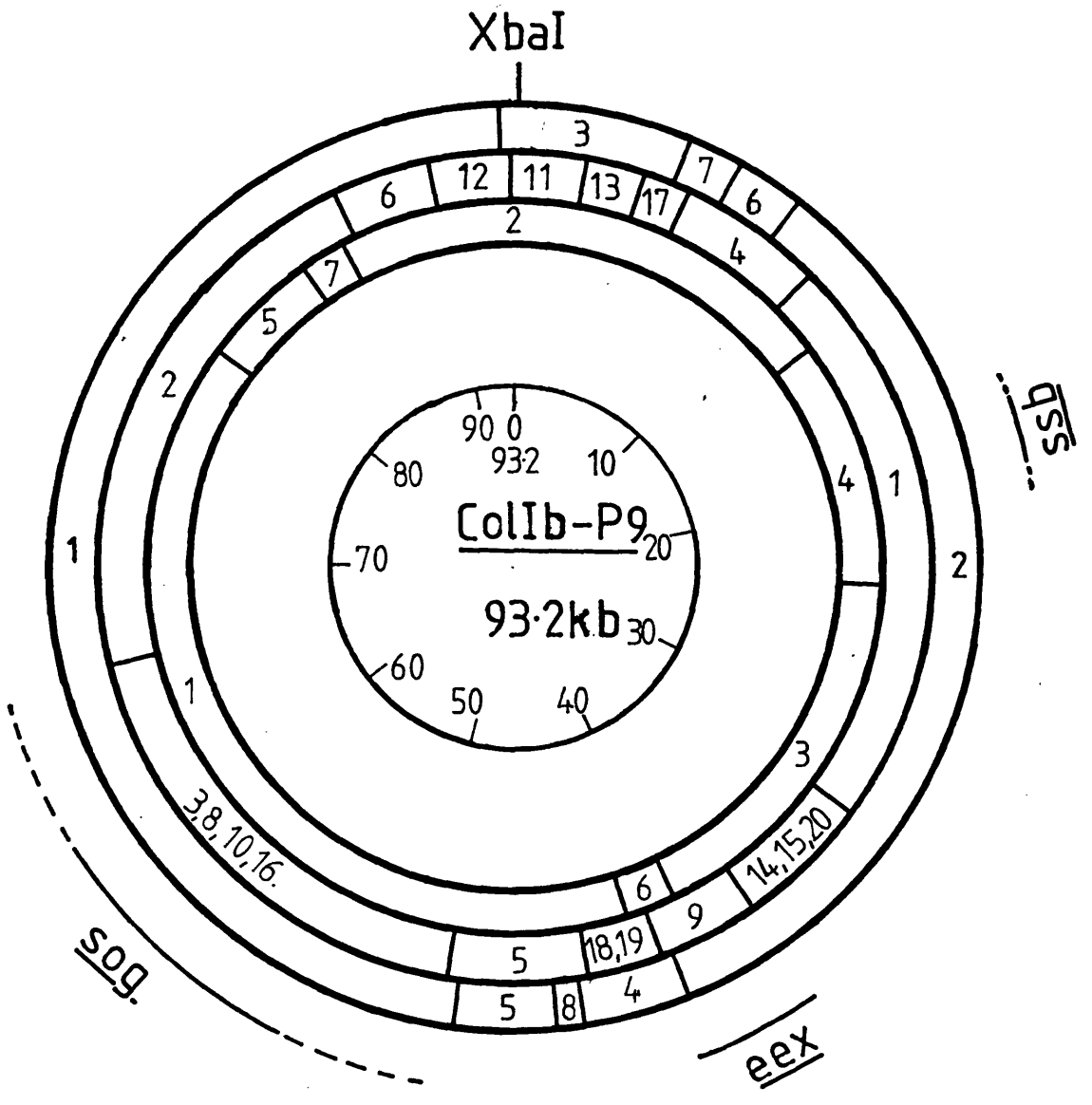
The $IncI_1$ conjugation system.

The two $IncI_1$ plasmids ColIb-P9 and R64, and the $IncI_1$+B plasmid R144 are all members of the I_1 complex (Hedges and Datta, 1973) and have been shown to be closely related by restriction analysis (Chabbert et al., 1979; Uemura and Mizobushi, 1982a; Furuichi et al., 1984; Hartskeerl et al., 1984). Bradley (1984) has shown that plasmids from this group determine serologically and morphologically indistinguishable pili and they have also been found to encode homologous plasmid primases (Dalrymple et al., 1982). R144, R64 and ColIb also specify closely related conjugation systems, as determined by their ability to mobilize a recombinant ColIb oriT plasmid (Wilkins et al., 1985). Therefore it may be presumed that findings for any one of these plasmids, with reference to the transfer genes, can be extrapolated to other members of the group.

The conjugation genes of the $IncI_1$ plasmids, like those of the IncFII plasmids, are naturally repressed. Before studies could be undertaken on these plasmids, derepressed mutants had to be isolated. ColIbdrd-1 was characterized by Meynell and Datta (1967) as a naturally occurring plasmid with increased fertility, but the nature of the

FIGURE 1.4 : Uemura and Mizobuchi restriction map of ColIb-P9.

The map kilobase coordinates are given relative relative to the single XbaI site. The inner circle represents the SalI cleavage map; the second circle, the EcoRI map and the outer circle the HindIII map (data taken from Uemura and Mizobuchi, 1982a). The fragments are numbered in decreasing size and throughout this thesis EcoRI, SalI and HindIII fragments of ColIb will be referred to by the letters E, S and H respectively, followed by the appropriate fragment number.
Also shown in the diagram are the approximate locations of the sog gene (Wilkins et al., 1981), the eex determinant (Chatfield et al., 1982) and the ssb gene (C. Howland, unpublished data).

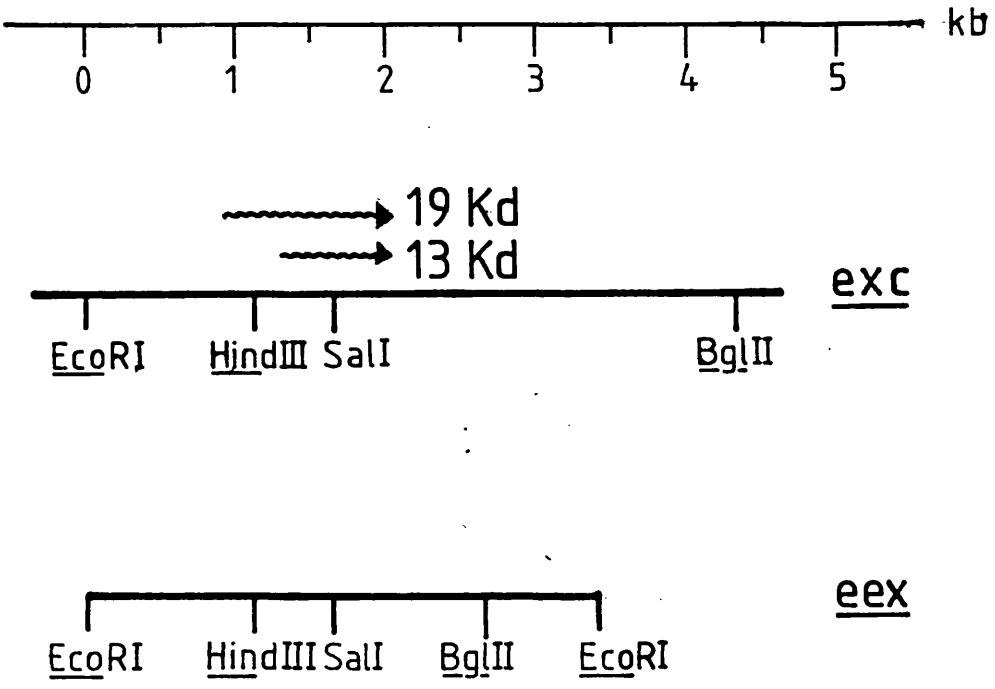

XbaI
3
7
6
12
11
13
17
6
2
4
7
5
2
ssb
90 0
93·2
10
80
4
1
ColIb-P9
20
70
2
93·2kb
30
1
60
50
40
3
3,8,10,16
14,15,20
6
9
18,19
5
4
5
8
sog
eex

mutation is not known. Bradley (1984) reported a method of selecting for drd $IncI_1$ plasmids using their ability to suppress bacterial temperature-sensitive dnaG mutations, but again this sheds no light on the control mechanisms involved in repression of the IncI conjugation genes. It has been suggested that the $IncI_2$ plasmids may have a two part repression system (Bradley, 1984).

The map of ColIb presented by Uemura and Mizobushi (1983a) contained some unordered EcoRI fragments, including the region where the 8 kb fragment known to contain the primase gene (sog) is located, see Figure 1.4. This gene has been shown to encode two antigenically related polypeptides of 240 Kd and 180 Kd, with only the N-terminal third of the larger poypeptide having primase activity (Wilkins et al., 1981; Boulnois et al., 1982). The 240 Kd polypeptide is thought to be able to provide RNA primers to initiate conjugative DNA synthesis in both donor and recipient cells (Chatfield et al., 1982), whilst the 180 Kd polypeptide acts following initiation of DNA transfer to promote efficient transmission of DNA (Merryweather et al., 1986b). What is notable about these two polypeptides is that during conjugation they are selectively transferred without proteolytic processing to the recipient cell, where the 240 Kd generates the RNA primers for complementary strand DNA synthesis (Chatfield and Wilkins, 1984; Merryweather et al., 1986b). Conjugative transfer of proteins has not been reported for any other conjugation system.

The exclusion determinant (eex) had been located to a 3.4 kb EcoRI fragment (Chatfield et al., 1982). This corresponds to E9 on the Uemura and Mizobuchi map (Figure 1.4). A comparison of the restriction sites of this fragment and those of the fragment containing the cloned exc determinant of R144 (Hartskeerl et al., 1983) suggests that this region may be common to both plasmids (see Figure 1.5). The exact location of the ColIb eex determinant is described in Chapter 6 and confirms that it

FIGURE 1.5 : THE ENTRY EXCLUSION REGIONS OF ColIb AND R144.



A comparison of the restriction maps of the cloned entry exclusion determinants of R144 (exc : Hartskeerl et al., 1983) and ColIb-P9 (eex : Chatfield et al., 1982; see Merryweather 1986). Arrows represent the 13 Kd and 19 Kd polypeptides encoded by exc (Hartskeerl et al., 1985b).

lies in an analogous position to the R144 exc gene and also indicates that ColIb exclusion is expressed independently of the other transfer genes.

The exclusion determinant is located within 10 kb of the sog gene on the plasmid. Therefore it seemed possible that the region between these two conjugation-related genes contains a block of transfer genes. The primase gene and exclusion determinants of the plasmid R144 have been located on this plasmid and are arranged analogously, with respect to the EcoRI map, to the ColIb sog and eex determinants (Hartskeerl et al., 1984). Using Tn1 mutagenesis it was shown that some of the DNA between the R144 sog and exc genes is required for transfer, and similarly insertions between exc and the oriT site (see Figure 1.6) also caused transfer deficiency. Accordingly the authors proposed a continuous block of transfer genes that cover the region between the sog gene and the origin of transfer (Hartskeerl et al., 1984), as indicated in Figure 1.6. The ColIb origin of transfer had been cloned on a 1.55 kb PstI fragment which originated from the 21.3 kb EcoRI fragment (Wilkins et al., 1985). Although the precise location of the PstI fragment was not known, the EcoRI fragment is found to cover an analogous region to that containing the R144 oriT site (see Chapter 3). Studies of R64 have located the sog gene of this plasmid to an 8.1 kb EcoRI fragment (Furuichi et al., 1984) which is contained within a core of DNA fragments, most of which are common to R64, ColIb and R144. It is thought that this region of the I_1 plasmids determines the conjugation genes.

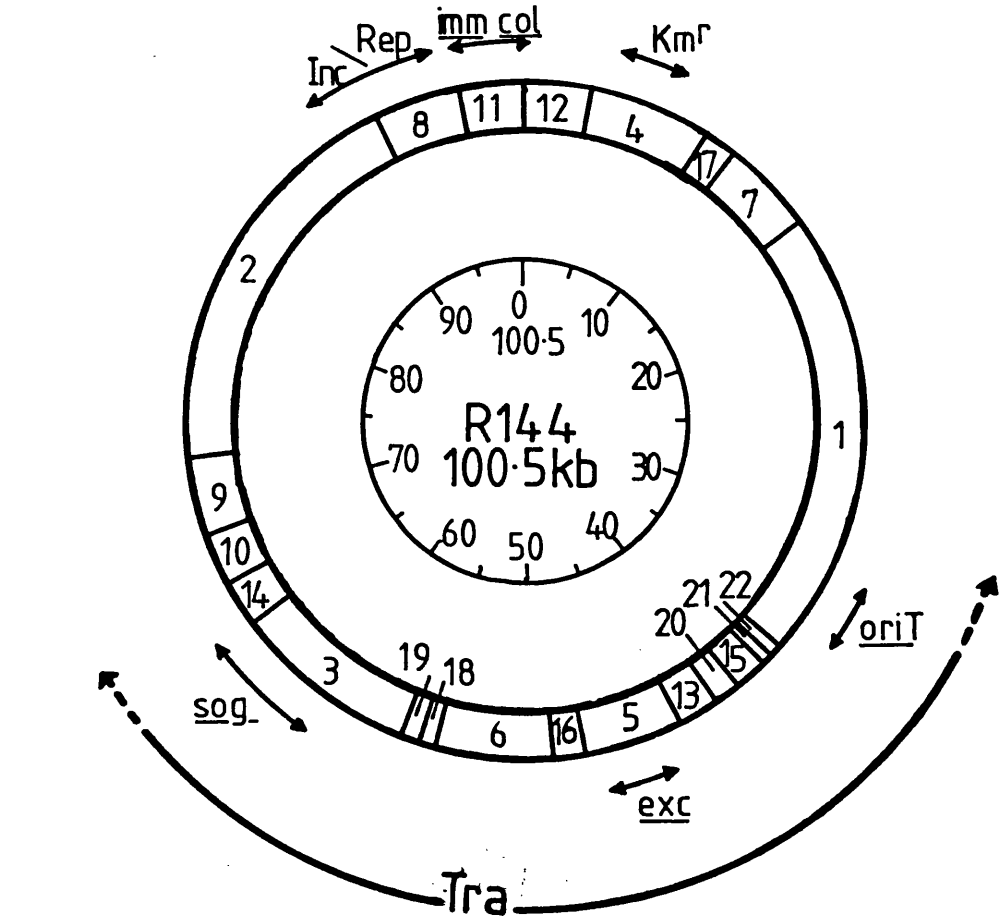
Unlike the conjugation systems so far described, plasmids of the I complex express two types of conjugative pilus (Bradley, 1984). Such a dual system was initially identified for the $IncI_2$ group of plasmids (Bradley and Coetzee, 1982) and was later shown to be determined by all plasmids of the I complex (Bradley, 1983). The pili specified by $IncI_2$ and $IncI_1$ plasmids are morphologically identical, but were found to be

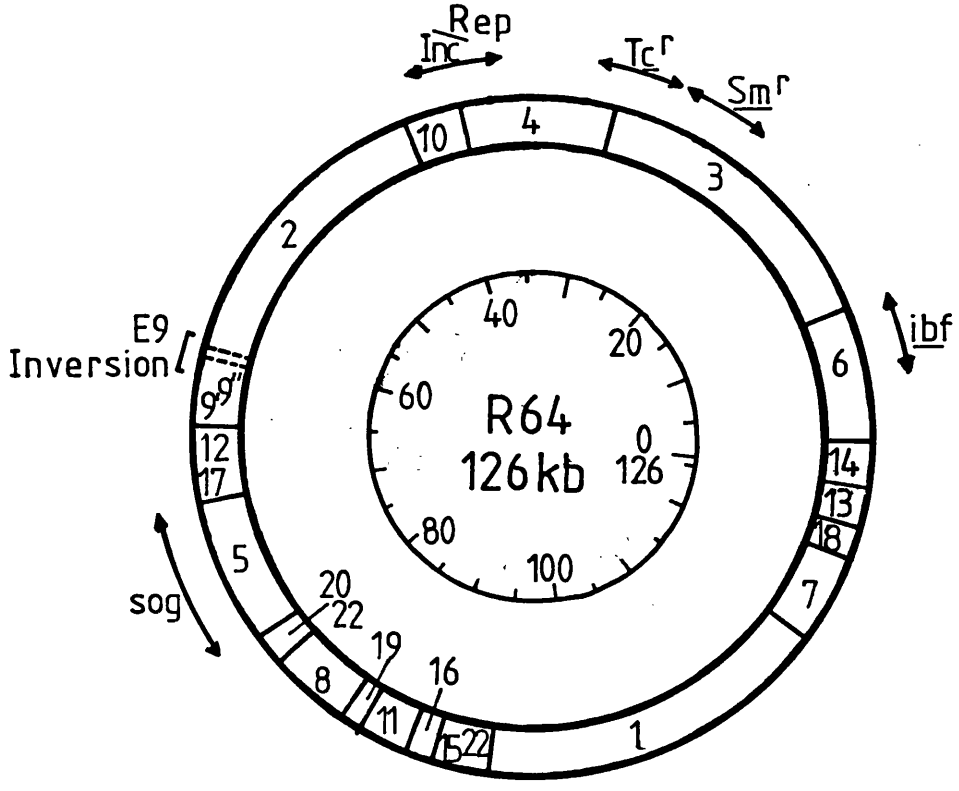
FIGURE 1.6 : EcoRI restriction maps of plasmids R144 and R64.

Both plasmids are drawn in approximately the same orientation as the standard map of ColIb used in this thesis.

R144 : Based on the map of Hartskeerl et al. (1984). The numbers within the ring represent EcoRI fragments. The region essential for replication (Rep) and the incompatibility determinant (Inc) are located at the top of the plasmid, along with the colicin Ib gene (col) and its immunity gene (imm). Also shown is the location of the kanamycin-resistance determinant (Km^R). The proposed tra region encompasses the R144 sog and exc genes and the oriT site.

R64 : Based on data given by Furuichi et al. (1984). The numbers within the ring represent EcoRI fragments. The plasmid is drawn so that the Inc and Rep determinants are in approximately the same position as those on the map of R144, but the map coordinates (inner circle) are given according to the convention of Furuichi et al. (1984). The tetracycline- and streptomycin-resistance determinants (Tc^R and Sm^R) are located in an equivalent site to the Km^R gene of R144. Also shown is the ibf determinant, which is located lower on this plasmid (as drawn) than are the ibf genes on ColIb. The location of the sog gene is shown, and above this the site of the inversion region of R64. This is in an analogous position, with respect to the sog gene and the Rep region, to the E8 inversion region of ColIb.


Rep
Inc
imm col
Km^r
R144
100·5kb
0
100·5
10
20
30
40
50
60
70
80
90
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
oriT
sog
exc
Tra


Rep
Inc
Tc^r
Sm^r
R64
126kb
0
20
40
60
80
100
126
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
22
E9
Inversion
ibf
sog

serologically unrelated by Bradley (1984). He also identified variation in the length of the thick pilus, and taken with the increased levels of thin pilus expression caused by treating I^+ cells with I pilus-specific antiserum (Lawn and Meynell, 1972), it seems that both types of I pilus may be able to retract.

The thick I_1 pilus has a diameter of 10.5 nm and is rigid with a pointed distal tip. The thin I_1 pili are flexible with a diameter of 6 nm and were found to aggregate together. Using cells resistant to phage which used the thin pilus as a receptor, Bradley (1984) showed that the thin pili were not required to mediate surface matings, but probably played a role in stabilization of mating aggregates in liquid media. It was demonstrated that they could perform this role equally well whether they were elaborated from the surface of either the donor or recipient cell (Bradley and Whelan, 1985).

Clustered Inversion region of R64.

When an EcoRI restriction digest of R64 was prepared, two unusually faint bands appeared on the gel, with a molar ratio of these DNA fragments to plasmid DNA of 1 : 2. Furuichi et al. (1984) proposed that an inversion region existed in R64 and by analogy with the other IncI plasmids, this would be found in the proposed tra region (see Figure 1.6). When this phenomenon was further investigated by restriction analysis of sub-cloned regions of R64 DNA, it was found that the event was not a simple flip-flop but involved a complex rearrangement of four segments of DNA which could invert with respect to the rest of the genome either independently or in groups, so that they were randomly arranged in any one plasmid. These inversions were found to be mediated by a plasmid-encoded recombinase encoded by the gene rci, located on the same EcoRI restriction fragment that was originally noted to be altered (Komano et al., 1986). A possible function for such a complex

recombination event is difficult to imagine, however in other bacterial systems flip-flop rearrangements of a single segment of DNA, mediated by site specific recombinases, have been shown to control biological switches (see Chapter 6). It is possible that the clustered inversion region of R64 may perform such a switching function to modulate the expression of either a very rare phenomenon or a hypervariable gene. A similar pattern had been observed when ColIb was digested with EcoRI and accounts for the anomaly in the numbering of EcoRI fragments by Uemura and Mizobuchi (1982a). What they took to be E7, a fragment which is not located on their map, was infact the larger form of E8 (Wilkins, unpublished data).

From all this data we now have some indication of in which region the tra genes of ColIb would be found. This supposition was supported by evidence that the genes known to be involved in functions other than conjugation had been mapped on the opposite side of the plasmid (see next section).

1.7 The functional organisation of ColIb-P9.

ColIb-P9 is characteristically identified by conferring on its host the ability to produce colicin Ib, a proteinaceous antibiotic (71 Kd: Varley and Boulnois, 1984). Addition of purified colicin to whole E.coli cells or cytoplasmic membrane vesicles has been shown to induce the formation of ion-permeable channels (Weaver et al., 1981a) and causes cell death by depolarisation of the membrane. The colicin acts by binding to a 74 Kd outer membrane receptor protein which may normally have some role in iron uptake (Konisky and Clowell, 1972: Bowles et al., 1983). After binding to this outer membrane site, it is thought that the colicin is transported to its site of action, the cytoplasmic membrane, by an undefined mechanism. Cells which express colicin Ib are immune to its effects. The immunity is mediated by a 14.5 Kd plasmid-encoded inner

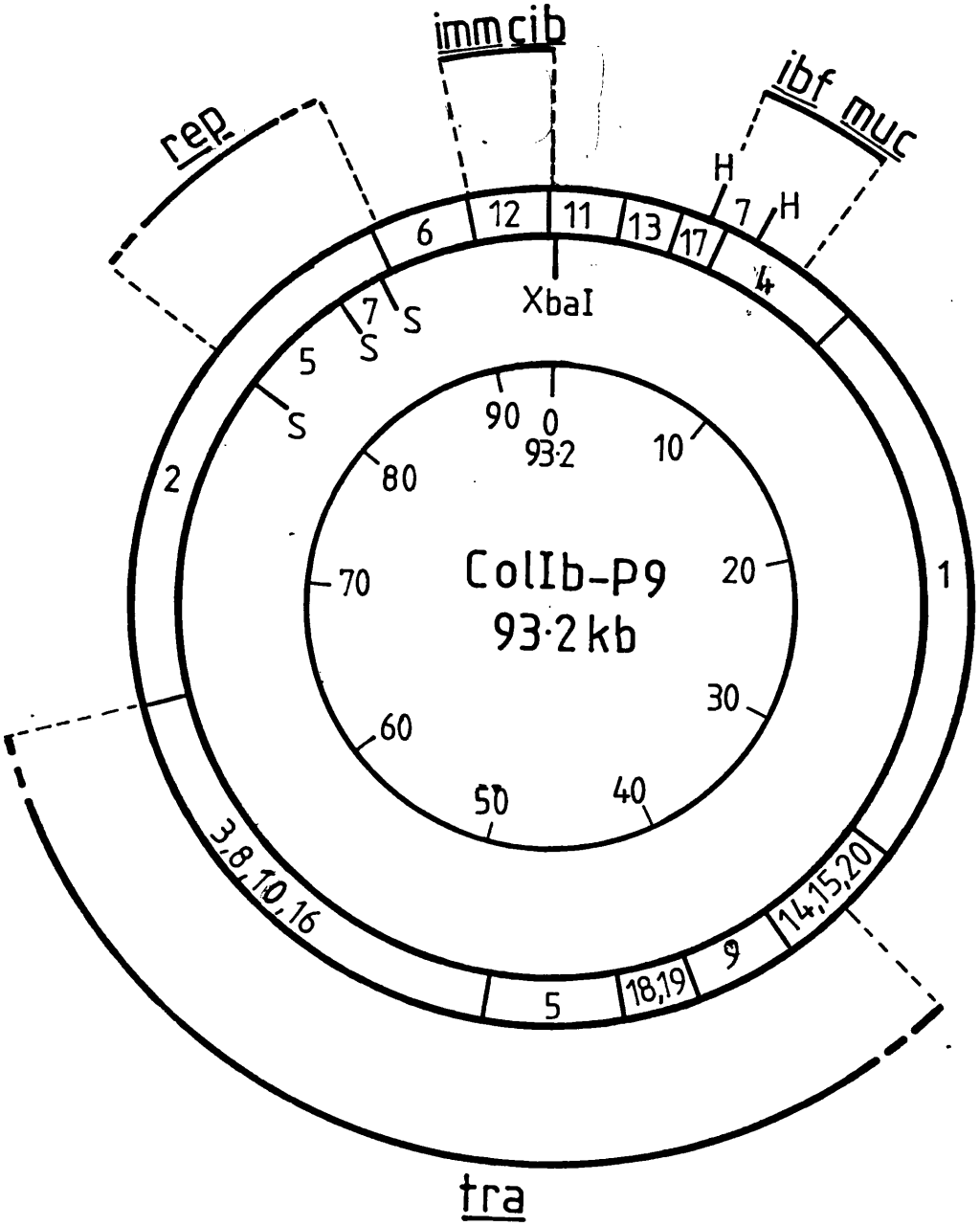
membrane protein which is thought to interact stoichiometrically with the colicin, after uptake, to inactivate it (Weaver et al., 1981b). The gene specifying colicin Ib (cib) has been cloned on a 2.7 kb EcoRI fragment (Boulnois, 1981). The location of this fragment on ColIb corresponds to the EcoRI fragment at coordinates 90.1 to 93.0 (E12) on the Uemura and Mizobuchi map. This position was confirmed by Gottlieb and Duckworth (1983) who mapped the large SalI fragment covering this region and located both the cib gene and its associated immunity gene (imm). The colicin gene is thought to be normally repressed, so that only ~0.01% of cells in a population express the gene. Evidence has suggested that the expression of cib can be induced by DNA damage, as part of the SOS response (Isaacson and Konisky, 1974; Pugsley, 1981; Glazebrook et al., 1983). In support of this Varley and Boulnois (1984) identifiied a putative SOS control sequence in the 5' region of the cib gene. However other factors are also involved in the regulation of the expression of this gene; it is also found to be subject to catabolite repression (as are many of the other SOS genes), and Glazebrook et al. (1983) also identified a plasmid-coded function on the BglII-A fragment of the IncI plasmid TP110 that could inhibit the expression of cib, when present in high copy number.

Adjacent to these genes, cloned on a 1.65 kb HindIII fragment are two related genes which cause abortive phage infection (ibfA and ibfB: Uemura and Mizobuchi, 1982b). Expression of these genes causes disruption of the uptake of T5-like phage, acting to block the synthesis of essential phage replication products (Moyer et al., 1972) by a process which may involve the host cell loci cmrA and cmrB (Hull and Moody, 1976).

ColIb has been shown to confer resistance to the lethal effects of UV radiation on host enterobacteria (Howarth, 1965) and to suppress chromosomal umuC mutations in E coli cells (UV protection and mutagenesis). Kopylov et al. (1984) isolated Tn5 mutants of ColIb which

FIGURE 1.7 : The functional organisation of ColIb-P9.

The numbers in the circle represent the EcoRI fragments of ColIb (Uemura and Mizobuchi, 1982a). Also shown are the single XbaI site and the positions of fragments S5, S7 and H7 (see Figure 1.4).
The location of the replication region (rep; Walia and Duckworth, 1983), the colicin gene (cib) and the colicin immunity gene (imm) (Boulnois, 1981), the abortive phage infection genes (ibf; Uemura and Mizobuchi, 1982b) and the muc genes (Kopylov et al., 1984). Also shown is the region thought to contain the transfer genes (see Section 1.7).


imm cib
rep
ibf muc
H
H
6
12
11
13
17
7
4
XbaI
7
S
S
5
S
90
0
93·2
10
80
70
20
ColIb-P9
93·2 kb
30
60
50
40
2
1
3,8,10,16
5
18,19
9
14,15,20
tra

had lost this ability, and the gene responsible was mapped to a 5.4 kb EcoRI fragment contained within the second largest SalI fragment. The phenotype was also expressed when this fragment was cloned in a low copy number vector. The imp genes (I group mutation and protection) have been best studied from the $IncI_1$ plasmid TP110. The position of the imp gene on this plasmid was located to a region 8.9 kb from the single XbaI site of the plasmid, on the opposite side to the colicin gene (Dowden et al., 1984). The gene was shown to be under lexA control and was transcribed towards the XbaI site. Further analysis identified two proteins (11 Kd and 51 Kd: Glazebrook et al., in press) involved in imp gene function and these were shown to share a similar genetic arrangement to that found for the related, but genetically distinct, UV repair and protection genes of the E.coli chromosome and IncN plasmids, umuDC and mucAB respectively (Perry et al., 1985). The ColIb imp genes, however, were shown to have a different mode of operation to the IncN muc genes (Kopylov et al., 1984).

As illustrated in Figure 1.7, all these functions are arrayed between coordinates 79.0 and 9.0, making this region apparantly analogous to the regions of R64 and RP4 which contain clustered resistance determinants, i.e. none of these genes are required either for maintenance or conjugal transfer of the plasmid. Several studies have indicated that the origin of vegetative replication (oriV) is also located in this region. From DNA hybridisation studies of several related ColIb plasmids, Walia and Duckworth (1983) deduced that oriV was located in or near the 5 kb SalI fragment of ColIb. They also used miniplasmids of ColIb to show that this region contained an incompatibility determinant. The most crucial evidence for the positioning of oriV comes from work by Furuichi et al. (1984) who used an 1.5 kb SalI fragment of ColIb to reconstruct a deleted rep region of R64 (region required for autonomous replication), indicating that the ColIb rep region must lie near coordinates 84.0 and

86.0. The location of the ColIb origin was determined in this work by isolating a SalI fragment of the plasmid which was able to replicate in a polA strain (see Chapter 3).

1.8 The structure of plasmid origins and the basis of incompatibility.

Sequencing data from the minimum rep regions of several low copy number plasmids has identified the presence of two common features; tandemly arrayed direct repeat sequences and an A + T rich region (pSC101: Churchward et al., 1983; IncP: Stalker et al., 1981; IncFII: Rosen et al., 1979; Light and Molin, 1981; IncQ: Haring et al., 1985; IncX: Stalker et al., 1979; F: Tolun and Helinski, 1981; Murotsu et al., 1981). The direct repeats have been shown to be required in cis for a functional origin of replication, deletion of multiples of these repeat sequences causing inactivation of the origin of R6K (IncX: Kolter and Helinski, 1982), F (Murotsu et al., 1984) and pSC101 (Yamaguchi and Yamaguchi, 1984). It has been proposed that the A + T rich region facilitates the melting of DNA strands to allow initiation of replication to occur, and that the direct repeats act as a binding site for a positively required replication protein. In vitro studies have shown that many of the plasmid encoded proteins required for initiation of replication bind specifically to the origin region, i.e. the π protein of R6K: Shafferman et al., 1982; O protein of λ: Tsurimoto and Matsubara, 1981; copB protein of R1: Light and Molin, 1982; repC protein of RSF1010: Haring et al., 1985; pSC101: Vocke and Bastia, 1983). It is thought that the binding of the protein to the origin either facilitates the assembly of the replication complex or alters the structure of the DNA in the origin region so that initiation may commence (Hobom et al., 1978, Riise and Molin, 1986).

Incompatibility is now regarded as a consequence of the replication control mechanisms of plasmids. Current models favour the idea that the rep proteins are competitively titrated by tandem repeat sequences near the origin and by those found in the incompatibility determinant of the plasmid (Perrson and Nordstrom, 1986; Chikiami et al., 1985; Chattoraj, 1984; Trawick and Kline, 1985). Levels of rep protein production are closely controlled by various feedback circuits involving both polypeptides and small RNA molecules (F: Bex et al., 1986; Seekle and Kline, 1984; R6K: Stalker et al., 1979, RK2: Thomas, 1986, IncFII plasmids: Brady et al., 1983; Nordstrom and Nordstrom, 1985; Light et al., 1985). When two low copy number plasmids with the same replication control mechanisms are present in the same cell, they are effectively treated as extra copies of one plasmid, and by titration of positively required factors by the extra copies of the repeated sequences, or by accumulation of repressor substance, replication is repressed until the copy number returns to normal. In the extreme case of F, which has a copy number of one, the plasmids segregate immediately. When the copy number is slightly higher the process may take a few generations, but the polarity is always such that the plasmid with the lowest copy number is retained.

This process of repression of replication when the copy number exceeds the norm, has been graphically demonstrated by linking high and low minireplicons together in a recombinant plasmid. Tsutsui and Matsubara (1981) used a miniF-ColE1 composite in a polA temperature sensitive strain. At the permissive temperature, replication was controlled by the ColE1 rep region and the copy number was high. Incoming λ phage which had the F rep region in place of its natural origin, could not replicate indicating that F replication was being repressed. When the temperature was raised and the ColE1 origin could no longer function (ColE1 requires functional DNA polymerase I to replicate;

Kingsbury and Helinski, 1973) initiation of replication from the F origin did not recommence until the copy number reached one or two. At this point the incoming recombinant phage could also replicate. A similar result was found by Cabello et al. (1976) using a pSC101-ColE1 composite plasmid.

More than one incompatibility locus has been identified for the F plasmid. A second, weaker form of incompatibility has been characterized as the incD locus, which is not required for automonous replication of the plasmid (Tolun and Helinski, 1981). This is thought to be a site involved in effective partitioning of the plasmid at cell division. The incompatibility is thought to arise through competition for sites on the host cell membrane to which the plasmid attaches (Gardener et al., 1982; Seekle et al., 1982). Evidence that the IncP plasmid RK2 is attached to the cell membrane during replication and that the plasmid-encoded trfA replication protein is located in the cell membrane, supports this model (Firshein et al., 1982; Firshein and Caro, 1984; Kornaki and Firshein, 1986). Evidence also exists that the E.coli chromosome origin is bound to the cell membrane (Kusano et al., 1984). Plasmids utilise host cell mechanisms during replication and therefore the site of the replication apparatus may be at the cytoplasm-membrane interface, and plasmid attachment to the cell wall may be involved in both replication and partitioning.

1.9 Aims of this study.

The description of the plasmid ColIb given in sections 1.6 and 1.7 was the starting point of this study. The location was known of many of the genes which were not involved in conjugation, and also two of the conjugation genes (eex and sog) had been isolated. Biologically, quite a wealth of information existed concerning the processing of plasmid DNA

during conjugation and the role of the sex pili, but futher understanding of these functions was now being hampered by a lack of knowledge of the genetics of the plasmid.

The initial aims were to construct a physical map of the plasmid and then to identify the position and organisation of the transfer genes with respect to that map. Much of this mapping was achieved by isolating extensive regions of ColIb DNA in recombinant plasmids that were more readily mapped using restriction enzymes (Chapter 3). This also allowed the identification of genes encoded on these fragments which in turn improved the functional map of ColIb.

One of the most useful tools available for investigating the organisation of bacterial genomes is the transposable element. The transposons used in this study were Tn5 and Tn1723 (Chapter 4). Tn5 is a composite transposon, the kanamycin resistance determinant being flanked by two IS50 elements, one of which encodes the transposition functions (see reviews by Reznikoff, 1982 and Berg and Berg, 1983). Tn1723 is a Tn3 like transposon encoding both transposase and resolvase functions within the main body of the transposon. This too carries a kanamycin resistance determinant which was inserted between the transposition genes and the end of the element (Schmitt et al., 1985). Both transposons have been shown to exert polar effects on genes in the same transcriptional unit downstream of the point of insertion (Berg et al., 1980, Schmitt et al., 1985), however some doubt has been expressed about the polarity of Tn5 in some transfer systems (see Manning et al., 1982 and Hansen et al., 1982), therefore care must be taken when interpreting the effects of these insertions. It was hoped that the polar effects of these insertions could be used to elucidate the operon structure of the transfer genes as well as indicating the physical location of individual genes. Difficulties encountered in isolating insertions in specific regions of interest required the use of two methods of generating

transposon insertions as described in Chapter 4. Complementation studies using these insertion mutants were made difficult by the phenomenon of plasmid incompatibility, but this was overcome to a certain extent using a series of cosmid recombinants containing large regions of ColIb plasmid DNA (see Chapter 7).

The results described in this thesis will be discussed at the end of each section. In the final chapter they are then brought together in to a model of the ColIb conjugation system which emphasises the differences between the $IncI_1$ transfer system and that of the F-like plasmids.

CHAPTER 2 : Materials and Methods

2.1 Bacterial Strains and Plasmids

Genotypic descriptions of bacterial strains used are given in Tables 2.1.1 and 2.1.2. A spontaneous nalidixic acid-resistant derivative of BW86 was isolated by plating 10 ml of cells (A_{600} = 0.35, Bausch and Lomb, Spectronic-20 spectrophotometer), concentrated in to 0.1 ml, on to L-agar plates which contained nalidixic acid at 50 $\mu l.ml^{-1}$. These were incubated over night and a few resistant colonies were found to have grown up against the background of cell death. One of these was purified to single colonies and checked to ensure it still had the same auxotrophic requirements as the parental bacteria, and that the BW86 still retained the temperature-sensitve *dnaG* phenotype.

A spontaneous λ-resistant mutant of BW85 was isolated by spotting 50 µl of a suspension of λ*vir* (1 x 10^6 $pfu.ml^{-1}$) on to 0.1 ml of an overnight culture of BW85 resuspended in 4 ml of SNA, spread on an L-agar plate. This was incubated for 36 hours before resistant colonies were picked against the background of lysed cells.

2.2 Media and Chemicals

2.2.1 Media.

Bacterial strains were routinely grown in nutrient broth 'E' (London Analytical and Bacteriological Media Ltd; 12.5 $gm.l^{-1}$) with aeration and on plates of Luria agar (L-agar) (10 $gm.l^{-1}$ Bacto-tryptone (Difco), 5 $gm.l^{-1}$ yeast extract (Difco), 5 $gm.l^{-1}$ NaCl (pH 7.0), 10.2 $gm.l^{-1}$ Bacto-agar (Difco). These media were supplemented with thymine (Sigma) at 20 $\mu l.ml^{-1}$ and with appropriate antibiotics for strains carrying plasmids (see Table 2.2.1). All antibiotics were supplied by Sigma except streptomycin (Glaxo).

TABLE 2.1.1 : Bacterial Strains.

Strain	Description or Genotype	Source or Reference
E.coli K-12		
BW85	*leu thyA deoB rpsL cir*	Boulnois *et al*. (1978)
BW86	*dnaG3 leu thyA deoB rpsL* Δ*(chlA-uvrB) cir*	Boulnois and Wilkins, (1979)
BW86N	*dnaG3 leu thyA deoB rpsL* Δ*(chlA-uvrB) cir nal*	This work
BW96	*leu deoA deoC tdk rpsL rpoB cir*	Boulnois and Wilkins, (1979)
BW97	*leu thyA deoB rpsL cir* Δ*(chlA-uvrB) cir nal*	Boulnois and Wilkins, (1979)
BW103	*recA1 leu deoB rpsL cir*	Merryweather *et al*. (1986a)
W3110	prototrophic	Bachmann (1972)
C600	*thr leu thi lacY1 supE44*	Bachmann (1972)
N1205	*recB21 recC22 sbcB15 thi-1 proA2 thr-1 leu-6 argE3 his-4 lacY1 galK2 ara-14 xyl-5 mtl-1 rpsL31*	R. Lloyd, Nottingham University.
JC411	*arg leu met his mal xyl lac rpsL*	Sullivan (1984)
JC411*polA*	*arg leu met his mal xyl lac rpsL polA214*	Sullivan (1984)

Genotypic symbols are defined in Bachmann (1983).

TABLE 2.1.2 : Plasmids.

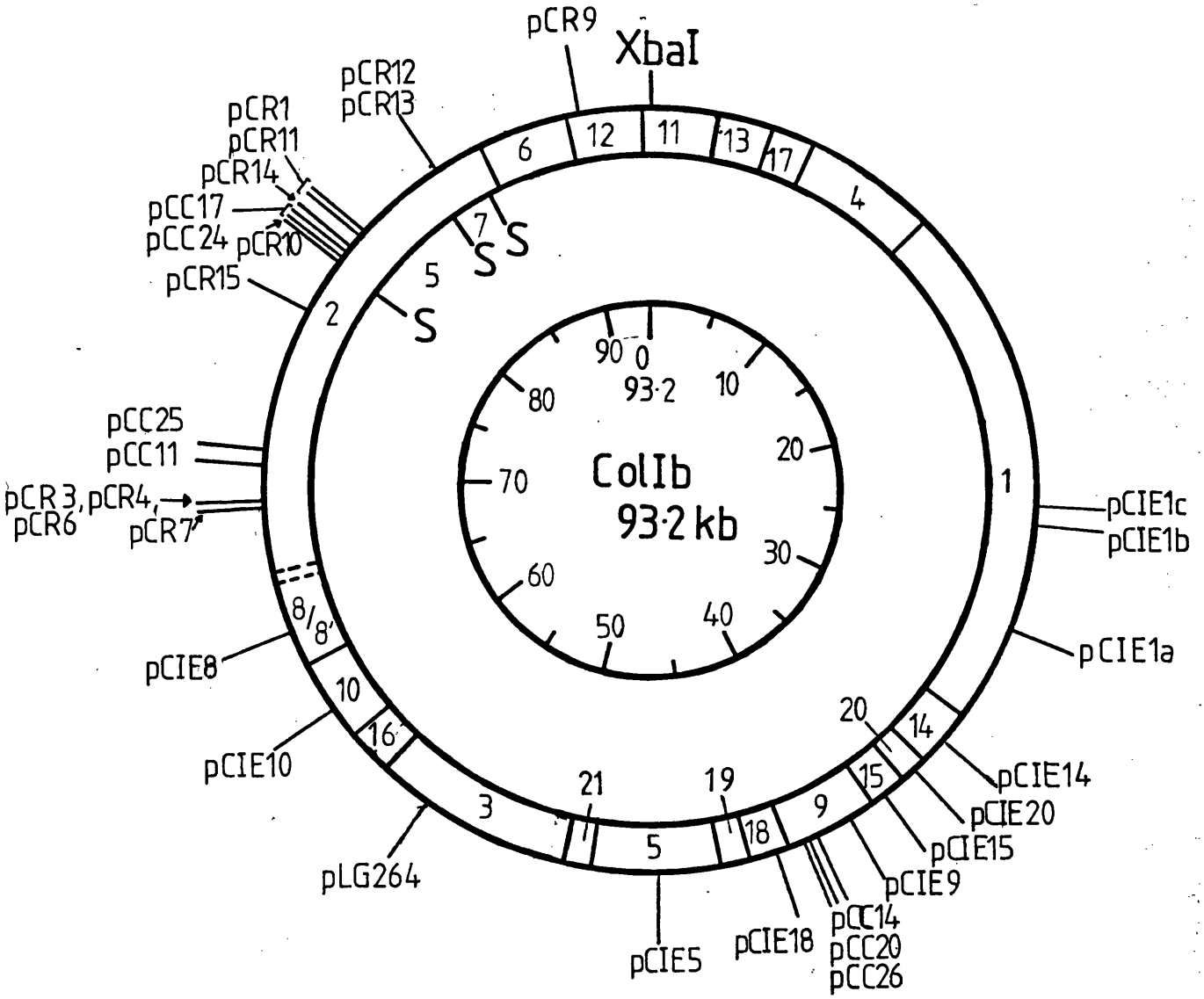
Plasmid	Description or Genotype	Source or Reference
$IncI_1$ plasmids		
ColIb-P9	93.2 kb, Cib^+, repressed for transfer	Laboratory stock
ColIb-P9drd-1	derepressed for transfer	G.G. Meynell (1969)
ColIb-P9drd-2	derepressed for transfer	Laboratory stock
pLG221	ColIb-P9drd-1 cib::Tn5 Km^R	Boulnois et al. (1981)
pLG250	pLG221sog-217 Km^R	Chatfield et al. (1982)
pLG264	ColIb-P9drd-2 sog::Tn5 Km^R	Merryweather et al. (1986b)
pLG269	ColIb-P9drd-2::Tn5 Tra^+ Km^R	Merryweather et al. (1986a)
pCR9	ColIb-P9drd-1 E12::Tn1723 Tra^+ Km^R	This work
pCR-plasmids	ColIb-P9drd-1::Tn1723 Km^R (see Figure 2.1.1)	This work
pCI-plasmids	ColIb-P9drd-1::Tn5 Km^R (see Figure 2.1.1)	This work
pCC-plasmids	ColIb-P9drd-2::Tn5 Km^R (see Figure 2.1.1)	This work
Vector plasmids		
pBR328	Ap^R Tc^R Cm^R	Soberon et al. (1980)

continued,.

TABLE 2.1.2, continued.

Plasmid	Description or Genotype	Source or Reference
R300B	IncQ Su^R Sm^R	Barth *et al.* (1981)
pTB92	R300B Su^R Km^R	Barth *et al.* (1981)
pGSS33	IncQ Ap^R Tc^R Cm^R Sm^R	Sharpe (1984)
Cos4	5.82 kb Ap^R Tc^R	C. Hadfield, Leicester Biocentre
Recombinant plasmids		
pLG215	pBR325Ω[Sog^+, *Eco*RI 8.3 kb] Ap^R Tc^R	Wilkins *et al.* (1981)
pLG262	pLG215 *sog*::Tn*5* Ap^R Km^R Tc^R	Merryweather *et al.* (1986b)
pLG252	pBR325Ω[Eex^+, *Eco*RI 3.4 kb] Ap^R Tc^R	Chatfield *et al.* (1982)
pLG2001	pBR328Ω[*oriT*, *Eco*RI 21.3 kb] Ap^R Tc^R	Merryweather *et al.* (1986b)
pLG2009	pBR328Ω[*oriT*, *Taq*Y1 ~860 bp] Ap^R	Merryweather *et al.* (1986b)
pCRS3	pBR328Ω[*oriT*, *Sal*I 15.9 kb] Ap^R Cm^R	This work
pCRS5	pBR328Ω[*oriV*, *Sal*I 5.0 kb] Ap^R Cm^R	This work
pCRS1a	pBR328Ω[Sog^+, *Sal*I 23.0 kb] Ap^R Cm^R	This work
pcos	Cos4Ω[~45 kb ColIb*drd-1* DNA] Ap^R	This work

FIGURE 2.1.1 : Summary map of the position of all the transposon insertions of ColIb described in this thesis.



The numbers in the circle represent the EcoRI fragments of ColIb (Uemura and Mizobuchi, 1982a). Also shown are the single XbaI site and the positions of fragments S5 and S7 (see Figure 1.4).

The site of each transposon insertion is indicated by a single line with the relative plasmid number which corresponds to the plasmid that contains an insertion at that point.

TABLE 2.2.1 : Concentrations of Antibiotics.

Antibiotic	Abbrieviation	Final Concentration
Ampicillin	Ap	100 $\mu g.ml^{-1}$
Chloramphenicol	Cm	25 $\mu g.ml^{-1}$
Kanamycin	Km	50 $\mu g.ml^{-1}$
Nalidixic acid	Nal	50 $\mu g.ml^{-1}$
Rifampicin	Rf	25 $\mu g.ml^{-1}$
Streptomycin	Sm	200 $\mu g.ml^{-1}$
Tetracycline	Tc	7.5 $\mu g.ml^{-1}$

Bacterial stains were kept on L-agar plates at 4°C but for long term storage, sterile 50% glycerol was added, to a final concentration of 25% (v/v) to an overnight culture of the strain grown with appropriate antibiotic selection. This was then stored at -20°C. Approximately 0.1 ml of this partially thawed culture could be used to inoculate 2 ml of nutrient broth in the absence of antibiotic.

Minimal Medium agar consisted of a solution of 42 mM Na_2HPO_4, 22mM KH_2PO_4, 18mM NH_4Cl, 8.5 mM NaCl, 22 mM glocose, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 3 μM thiamine HCl, which contained 15 $gm.l^{-1}$ agar (Oxoid N°2). Appropriate amino acids were provided (20 $\mu g.ml^{-1}$ final concentration, except leucine 40 $\mu g.ml^{-1}$) when testing for auxotrophic mutants. SGC broth contained the same salts as those supplied in minimal agar but was supplemented with 2.5 $gm.l^{-1}$ Casaminoacids (Difco).

Phosphate buffer (50mM Na_2HPO_4, 22 mM KH_2PO_4, 68 mM NaCl, 0.4 mM $MgSO_4$) was used for serial dilution of cells or resuspension of single colonies.

2.2.2 Radiochemicals.

Radiochemicalswere purchased from Amersham International.
[methyl-^{3}H]thymine: specific activity was 1.92 $TBq.mmol^{-1}$, at a concentration of 37 $MBq.ml^{-1}$.
[^{32}P-α]dCTP: specific activity was 11.1 $TBq.mmol^{-1}$, at a concentration of 3.7 $MBq.ml^{-1}$.

2.3 Phenotypic Characterisation of Bacterial Strains.

2.3.1 Determination of Growth Requirements and resistances

Bacteria were streaked to single colonies on minimal media plates containing thiamine-HCl, with the addition of thymine and aminoacids as

appropriate, at the concentrations given in Section 2.2.1.

Strains carrying antibiotic resistance determinants were tested on L-agar plates containing antibiotics or where convenient by placing multodisks (30-44K, Oxoid) on an L-agar plate that had been spread with a lawn of the strain to be tested.

2.3.2 Sensitivity to Bacteriophage

Sensitivity to bacteriophage was tested qualitatively by cross streaking overnight cultures of the cells across a suspension of the phage which had been dried on to a non-selective L-agar plate.

A more quantitative measure was achieved by spotting 10 µl of a serial dilution of a phage stock on to 0.1 ml of a log-phase culture (A600 = 0.35) spread in 4 ml of SNA on a non-selective L-agar plate. The number of individual plaques could then be counted at each dilution.

2.3.3 Production of Colicin Ib

Production of colicin Ib was detected by spotting the test strain on to a L-agar plate and incubating for 6 to 14 hours. The plates were then exposed to chloroform vapour for 20 minutes to kill the test colonies. The chloroform was allowed to evaporate away for a further 30 minutes before the plates were overlaid with 4 ml of SNA containing 0.1 ml of a 1 in 20 dilution of an overnight culture of a colicin sensitive indicator strain. After 5 to 6 hours incubation at 37°C, a clear halo of killing around the test strain indicated the production of colicin Ib.

2.3.4 Suppression of <u>dnaG</u>

Suppression of the temperature sensitive <u>dnaG3</u> phenotype of host strains carrying ColIb plasmids, was tested by their ability to form colonies at the non-permissive temperature, 40°C. An overnight culture

of the test strain was diluted to an A_{600} of 0.35 and then serially diluted to give single colonies when 0.1 ml was plated on L-agar plates. The cell suspension was plated out in duplicate, one of the two plates being prewarmed to 42°C. The pairs of plates were then incubated for 24hrs, at 30°C and the prewarmed plate at 40°C. Suppression of dnaG3 temperature sensitivity (colony-forming ability) was measured as the percentage survival of the strain at the non-permissive temperature.

2.3.5 Measurement of sog mediated DNA synthesis

This method relies on the plasmid carrying strain having a temperature sensitive dnaG mutation and is based on the method of Wilkins et al. (1981). Cells were diluted from an overnight culture in SGC broth supplemented with 3 $\mu m.l^{-1}$ thymine and 200 $\mu l.ml^{-1}$ deoxyguanidine (Sigma), to an A_{600} of approximately 0.05 in 5 ml of the same medium. An accurate measurement of the A_{600} was then determined using a Gilford 300N Micro-sample spectrophotometer. The cultures were shaken at 30°C until the A_{600} reached ~0.4 (maximum value), at which point again the optical density was determined accurately. The cells were then transferred to a 41°C water bath for 5 minutes to inactivate host dnaG activity. 1.5 ml of the heat shocked culture was transferred in to a flask containing 1.5 ml of prewarmed supplemented SGC medium containing 22.5 µCi of ^3H-thymine. The cultures were shaken for 90 minutes at 41°C before 1 ml samples were taken and added immediately to 2 ml of ice cold TCA-thymine solution (70 $mg.ml^{-1}$ TCA, 5 $mg.ml^{-1}$ thymine). Acid insoluble material was allowed to precipitate for 30 minutes at 0°C before filtering on to nitrocellulose discs (Whatman, 25 mm diameter, 0.45 µm pore size), which had been presoaked in 200 $mg.ml^{-1}$ thymine solution. Acid soluble fractions were removed by extensive washing with boiling water before the filters were dried under a 100W lamp. 1 ml of non-aqueous scintillation fluid (5 gm 2, 5-diphenyloxazole

[PPO, Fisons] and 0.3 gm 1, 4-di-2(4-methyl-5-phenyl oxazolyl) benzene [POPOP, Fisons] per litre of toluene [Fisons]) was added to each filter in a scintillation vial. Radioactivity incorporated into the acid insoluble fraction was measured using a Packard 3255 liquid scintillation counter (^3H channel).

2.3.6 Nuclease Assay.

This assay employed the rapid mini-preparation method of Klein et al., (1980). 10 ml of an overnight culture of cells were resuspended in 0.5 ml of 50 mM Tris-HCl (pH 8), 5 mM EDTA and 0.25 ml of this was dipensed into each of two eppendorf tubes. All further stages were performed in duplicate. 50 µl of lysozyme solution (10 $mg.ml^{-1}$) was added to the cells which were then incubated at room temperature for 15 minutes. The cells were lysed by adding 0.5 ml of buffer-equilibrated phenol solution (100 gm phenol [BDH Chemicals Ltd.], 0.1 gm 8-hydroxyquinoline [BDH], 4 ml isoamylalcohol [Fisons] in 100 ml chloroform [Fisons], stored under 10 mM Tris-HCl, pH 7.5). Both phases were mixed well before centrifuging at top speed in an MSE Microcentaur centrifuge (microfuge) for 15 minutes. After this the aqueous top layer was removed and added to an equal volume of fresh phenol solution and the process repeated. The aqueous layer was removed to a clean eppendorf tube and residual phenol extracted twice by mixing with 24 : 1 chloroform : isoamylalcohol, centrifuging in the microfuge for 3 minutes and removing the clean DNA solution. The DNA was precipitated by adding one tenth volume 2 M sodium acetate solution (pH 5.6) and two volumes of absolute ethanol. After 5 minutes incubation at room temperature, the DNA was pelleted for 5 minutes in the MSE microfuge. The DNA pellet was resuspended in 50 µl of water, and approximately 20µl of this was run on a 0.7% (w/v) agarose gel (Seakem) at 30V for 16 hours.

2.3.7 Enzyme linked immunoabsorbant assay for synthesis of thick I_1 pili (ELISA).

Bacteria were grown from a fresh overnight culture for approximately four generations (until the A_{600} reached 0.6). 3 ml of cells were then shaken vigorously in a mating interrupter for 30 seconds, before the cells and debris were removed from the supernatant by centrifuging for 10 minutes in a MSE benchtop centrifuge at top speed. 5 µl of the supernatant were then spotted on to a strip of nitrocellulose and dried in with a hairdryer. The filter was incubated over night in Tris-saline (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% (w/v) BSA) at 4°C.

The filter was then treated with a 1 in 100 dilution of anti-pilus rabbit IgG (D. Bradley, 1983), for 90 minutes at room temperature. This was removed by washing several times in fresh Tris-saline before treating the filter with a 1 in 1000 dilution of goat anti-rabbit IgG linked to horse radish peroxidase (Bio-Rad) for 90 minutes (both antibodies were diluted in Tris-saline). Excess antibody was removed with several washes in Tris-saline before developing the colour reaction in 60 ml of Tris-saline containing 10 mls of 4-chloro-1-naphthol [Sigma] solution (3 $mg.ml^{-1}$ in methanol) and 30 µl of H_2O_2, for approximately 30 seconds. The filter was then rinsed in distilled water before drying. Strains which expressed thick I_1 pili produced a dark purple spot on the filter, whereas pilus negative strains produced only a faint background colouring (see Figure 5.02).

2.4 Bacterial matings

2.4.1 Mating in liquid media.

This method was used to measure transfer deficiency, frequency of mobilization, surface exclusion and incompatibility. Both donor and recipient strains were grown with aeration to A_{600} = 0.35. Donors and

TABLE 2.5 : Standard Molecular Weight DNA Markers.

λ cI_{857} Sam7 x HindIII (kb)	λ cI_{857} Sam7 x HindIII + EcoRI (kb)	λ x XhoI (kb)
23.13	21.22	33
9.4	5.15	10
6.56	4.97	
4.36	4.27	
2.32	3.53	
2.03	2.03	
0.56	1.91	
	1.58	
	1.38	
	0.95	
	0.83	
	0.56	
	0.13	

Figures taken from Sanger et al. (1982).

recipients were then mixed in a 1:1 ratio (final volume of 2.5 ml) in a 50 ml sterile flask and incubated at the permissive temperature with gentle swirling (speed N°3 in a New Brunswick gyrotory waterbath). After 1 hour, 0.1 ml samples of this were taken and appropriate dilutions were plated on media selective for transfer of plasmid(s) into the recipient strain.

2.4.2 Mating on Solid Media

This method was used to measure the efficiency of transfer in the absence of the thin flexible I pilus. Donor and recipient cells were grown from A_{600} = 0.05 to 0.35. Equal volumes of each (0.75 ml) were mixed and then pipetted on to nitrocellulose discs (Whatman, 25 mm, 0.45 µm pore size) using low house suction. The filters were dried under high suction for 15 seconds and then placed on to a non-selective prewarmed L-agar plate with the bacteria uppermost. These were incubated for 1 hour at the permissive temperature before the cells were resuspended from the filter in 5 ml of phosphate buffer by vortexing vigorously for 30 seconds. The cells were plated out on to selective agar at appropriate dilutions to give single transconjugant colonies.

2.5 DNA Manipulations.

2.5.1 Minipreparation of Plasmid DNA.

This method was used for all routine preparation of DNA for cloning, restriction analysis and transformation. The method is based on that of Ish-Horowicz and Burke (1981).

1.6 ml of an overnight culture of cells was centrifuged in an MSE microfuge for 30 seconds. The supernatant was removed carefully before resuspending the cells in 100 µl of 25 mM Tris-HCl (pH 8), 10 mM EDTA, 50 mM sucrose solution. They were incubated on ice for 5 minutes and

then 200µl of 0.2 M NaOH, 1% SDS solution were added. This was mixed in well by gently inverting the tube and the incubation was continued for a further 5 minutes on ice. Finally, 150 µl of 3 M potassium acetate solution (pH 4.8) were added, mixed in thoroughly and the incubation was continued for 5 minutes. The resultant lysate was centrifuged at top speed in an MSE microfuge for 1 to 2 minutes and then the supernatant was transferred to a clean eppendorf and phenol extracted and chloroform extracted twice (see section 2.3.6). The DNA was precipitated by adding one tenth volume 2 M Sodium Acetate solution (pH 5.6) and two volumes of absolute ethanol, incubating at room temperature for 5 minutes and pelleting the DNA for 3 minutes in the MSE microfuge. The pellet was resuspended in approximately 30 µl of distilled water and stored at 4°C. The DNA was treated with RNaseA (Sigma) (250 $\mu g.ml^{-1}$ final concentration) on use.

2.5.2 Uemura and Mizobuchi large scale preparation of plasmid DNA.

This method could only be reliably used for plasmids which were defective in nuclease expression and is based on that given by Uemura and Mizobuchi (1982a).

A volume of 250 ml of nutrient broth was inoculated with 8.3 ml of an overnight culture of the plasmid containing strain, and grown with selection until the cells reached late exponential growth (A_{600} = 1.0). The cells were then pelleted in a GS3 Sorvall rotor at 5 K for 10 minutes at 4°C in a Sorvall RC-5 centrifuge. They were then washed in one quarter volume (62.5 ml) of 50 mM Tris-HCl (pH 8), before centrifuging as before. The pellet was then resuspended in 6.25 ml of 25% (w/v) sucrose in 50 mM Tris-HCl (pH 8) and transferred to 20 ml nalgene tubes (Du Pont). To this was added 1.25 ml of 5 $mg.ml^{-1}$ lysozyme (Sigma) solution in 250 mM Tris-HCl (pH 8) and then incubated on ice for 5 minutes. After this, 2.8 ml of 500 mM EDTA (pH 8) were added and the

incubation continued for 5 minutes, with gentle swirling from time to time. The cells were completely lysed by adding 10 ml of detergent mixture (1% (w/v) Brij [Ciba-Geigy], 0.4% (w/v) sodium deoxycholate [Ciba-Geigy], 50mM Tris-HCl (pH 8), 125 mM EDTA) and incubating on ice for a further 10 minutes. The cell debris was removed by centrifuging the lysate in a SS34 Sorvall rotor for 40 minutes at 18 K at 20°C in a Sorvall RC-5 centrifuge.

The supernatant from this was decanted into a clean nalgene tube and sodium N-lauroyl sarcosinate (Sigma) powder added to a final concentration of 1% (w/v) and allowed to dissolve at 30°C for 10 minutes with gentle swirling. The DNA was then precipitated by chilling the solution before adding one tenth volume of 5M NaCl (to a final concentration of 0.5 M) and PEG 6000 (Fisons) powder to a final concentration of 10% (w/v). This was left to stand at 4°C over night. The DNA was then pelleted by centrifuging for 10 minutes at 7 K in a Sorvall SS34 rotor.

The pellet was dissolved in 1.1 ml of TES buffer (50 mM Tris-HCl (pH 8), 50 mM NaCl, 5 mM EDTA) and layered on top of 4 ml of CsCl mix (40 gm CsCl (Fisons) in 25 ml TES, 2 ml of 5 $mg.ml^{-1}$ Ethidium bromide solution, 3 ml of 250 $mg.ml^{-1}$ sarkosyl in TES, RI = 1.3990 - 1.4000) in a Beckman quickseal Vti65 tube. The tubes were then balanced using the CsCl mix before sealing. The plasmid DNA was then separated from the chromosomal DNA by centrifuging for 16 hours at 50 K at 15°C or for 3 hours at 55 K, 15°C in a Beckman L5-65 centrifuge. Once the gradient had equilibrated , the lower DNA band was identified under a UV lamp and extracted using a syringe. The ethidium bromide was removed by extraction with an equal volume of CsCl saturated isopropanol. The solution was then dialysed against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and finally the DNA was concentrated by ethanol precipitation. The pellet was resuspended in ~250 µl of distilled water and stored at 4°C.

2.5.3 Restriction analysis.

DNA prepared by either of the two methods described in Sections 2.5.1 and 2.5.2, were digested with various restriction enzymes in the buffers recommended by the manufacturer. Digests were standardly performed in a volume of approximately 25 µl in the presence of 10 mM spermidine (Bouche, 1981) and 250 $\mu g.ml^{-1}$ RNaseA, and were incubated at 37°C for 1 hour. DNA samples were mixed with one sixth volume of loading buffer (80 mM Tris-HCl (pH 7.5), 100 $mg.ml^{-1}$ glycerol, 0.01 $mg.ml^{-1}$ bromophenol blue).

Restriction fragments were separated by size on HGT Seakem agarose horizontal slab gels of 0.5 - 1.5% (w/v) in TE buffer (40 mM Tris-acetate (pH 7.4), 1 mM EDTA). Separation was achieved by running the gels over night at 30V in TE buffer containing 6 $\mu g.ml^{-1}$ ethidium bromide. The restriction fragments were visualised on a short-wavelength UV transilluminator and photographed using Kodak PlusX (4147) film (15 seconds exposure, f4.5). The size of restriction fragments was determined by comparison with standard molecular weight markers (see Table 2.5).

2.5.4 Cloning Procedures.

The vector DNA and the plasmid DNA to be ligated were cut with appropriate restriction enzymes and then phenol and chloroform extracted as described in Section 2.3.6 and ethanol precipitated (see Section 2.3.6) to remove these enzymes. The concentration of each of the digested DNAs was then determined approximately by, comparison to a known concentration of DNA, by running a small sample on an agarose gel. The restricted vector and plasmid DNA was then mixed to give a fragment ratio of 1 : 3 plasmid DNA : vector DNA, such that the total DNA in the reaction was 0.25 µg. The total volume of the reaction was kept to 50 - 100 µl. To the DNA was added one tenth volume of 10x ligation buffer

(66 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM ATP, 10 mM DTT, 50 $\mu g.ml^{-1}$ BSA (BRL, nuclease free), stored at -80°C) and an appropriate volume of distilled water. Finally, 0.2 units of T4 ligase (Biolabs) were added. The ligation reaction was incubated overnight at 15°C before running a small aliquot (~50 ng of DNA) on an agarose gel to check that ligation of fragments had occurred. The ligation mixture could then be used to transform a suitable host strain.

2.6 Strain manipulations.

2.6.1 Transformation.

The method described here is based on that of Cohen et al., 1972. 10 ml of cells were grown in nutrient broth with aeration from an A_{600} of 0.05, through three generations to an A_{600} of 0.35. If the strain to be transformed already contained a plasmid, the cells were grown in the presence of antibiotic selection for that plasmid. They were then diluted back to A_{600} = 0.05 and again grown for three mass doublings so that most of the cells were in exponential phase growth. The cells were then pelleted by centrifuging at 4 K at 0°C in an MSE Chillspin centrifuge for 8 minutes. They were washed in 5 ml of ice cold 100 mM $MgCl_2$ and then pelleted as before. They were then resuspended in 5 ml of 100 mM $CaCl_2$ (0°C) and incubated on ice for up to 1 hour. After this the cells were pelleted as described and resuspended in ice cold 100 mM $CaCl_2$ to give 250 µl of cells per transformation, up to 1ml total volume. 250 µl of competent cells were used per transformation and up to four transformations could be perfomed per 10 ml of starting culture. To achieve a 10 fold increase in the efficiency of transformation, the cells were held at this stage, overnight, packed in ice. However if the transforming DNA was a relatively small (15 kb or less), this step was unnecessary.

The cells were transferred to a sterile eppendorf tube and the plasmid DNA (0.25 - 0.5 μg) was added to them and mixed in well. This was allowed to stand on ice without being disturbed for 1 hour. The cells were then heat shocked at 42°C for 3 minutes, again without disturbing the pellet, and returned to ice for a further 10 minutes. After this, the transformation mixture was added to 5 ml of prewarmed (30°C or 37°C) nutrient broth and the newly acquired plasmid genes allowed to express for 1 hour. The cells were then concentrated into 0.3 ml of phosphate buffer before plating out on selective media to give single transformant colonies.

2.6.2 Preparation of plating cells for phage infection.

The strain to be infected was diluted from an overnight culture to an A_{600} of 0.05 in nutrient broth, with any necessary selection for plasmids carried by that strain. The bacteria were then grown to stationary phase (A_{600} = 0.6) and resuspended in 80% final volume of λ-buffer (10 mM $MgSO_4$, 6 mM Tris-HCl (pH 7.4), 50 $\mu g.ml^{-1}$ gelatin). These could be stored before use for up to 7 days at 4°C.

2.6.3 Preparation of phage Iα, PR64FS and λ::Tn*5*.

Phage stocks were amplified from existing phage suspensions by mixing 0.1 ml of the phage suspension (~1 x 10^8 $pfu.ml^{-1}$) with 0.4 ml of cells of a suitable strain. For Iα and PR64FS this was BW85(pCR9) in exponential phase growth (grown form A_{600} = 0.05 to 0.35). For λ::Tn*5* C600 was grown to stationary phase (A_{600} = 0.6) and resuspended in 80% of initial volume λ-buffer (see Section 2.6.2). The infections were allowed to proceed for 10 minutes at room temperature before adding 3.5 ml of SNA and pouring this over a moist L-agar plate supplemented with 20 $\mu g.ml^{-1}$ thymine. This was incubated over night and plaques allowed to form. The SNA was then scraped off the surface of the plate

and transferred to a 25 ml Sorvall tube and macerated using a sterile spatula. The agar was removed by centrifuging for 10 minutes at 10 K in an SS34 Sorvall rotor in a Sorvall RC-5 centrifuge, at room temperature. The supernatant was decanted in to a clean Sorvall tube and the process repeated.

The resultant phage suspension was sterilised by adding one tenth volume of either chloroform (λ::Tn*5*) or ether (Iα and PR64FS) (not more than 0.5 ml) and shaking vigorously. After standing for 20 minutes the solvent was removed with a pasteur pipette and by aeration for 20 to 30 minutes to remove residual amounts of the solvents. Before use, the phage was titred on a sensitive strain using the same method was used as that used to amplify the phage, but using lower dilutions of phage stock so that individual plaques could be counted. λ::Tn*5* was also titered on W3110 (*sup*^{o}) to determine what proportion of the phage had lost the Oam mutation (generally about 0.000014%).

2.6.4 Abortive phage infections with λ::Tn*5*.

An overnight culture of the bacterial strain to be infected was grown, with selection for any plasmid carried by that strain, from an A_{600} of 0.05 to 0.6. $MgCl_2$ was added to a final concentration of 10 mM. 0.4 ml of cells were mixed with 0.4 ml of phage at an appropriate dilution to give a multiplicity of infection of 100 (A_{600} of 0.6 = ~ 3.4×10^8 cells per ml). The infection was allowed to proceed for 30 minutes at room temperature before the mixture was plated out on L-agar plates containing 50 $\mu g.ml^{-1}$ kanamycin plus selection for any plasmid markers. The plates were incubated over night at the permissive temperature for the host strain.

2.7 Cosmid cloning.

Cosmid vectors, for cloning large fragments of DNA were first developed by Collins and Hohn (1978) and contain both a plasmid origin of replication and the cos site of phage λ so that features of the replication of both of these systems can be utilised. The cosmid vector is optimally about 4 - 6 kb and therefore can accomodate up to 45 kb of inserted DNA.

Partial digests containing approximately 2.5 µg of ColIbdrd-1 DNA were performed for 5, 10 and 15 minutes using the restriction enzyme Sau3A, diluted to 0.5 units per µl (total volume of the reaction was 40 µl). After this time the reactions were stopped by heat inactivating the enzyme at 65°C for 5 minutes. 5 µl samples were run out on a 0.5% agarose gel to check that the digestion had not proceeded too far (the DNA was run next to λ phage DNA cut with XhoI standard: 33 kb and 10 kb bands). An aliquot of 0.5 µg was taken from each digest and added to an equal amount of Cos4 vector DNA (Lehrach and Reedy [European Molecular Biology Laboratory, Heidelberg, FRG] unpublished, obtained from Dr. C. Hadfield, Leicester Biocentre.) which had been cut with BamHI. These two were ethanol precipitated together before resuspending in 4.5 µl of distilled water. They were then ligated together by adding 0.5 µl of 10x ligase buffer and 1 µl of T4 ligase (1 unit). This was incubated over night at 15°C. The ligation reaction was checked by making the volume of the mixture up to 22 µl and running 3 µ on a 0.5% agarose gel. Three vector bands were visible (1.8 kb and 4 kb vector arms and a 6 kb band that corresponded to these ligated together) and a high molecular weight smear correspoding to the cloned fragments. 5 µl of the ligation mix was added to the top of an aliquot of frozen packaging extract (-80°C, prepared by G. Walker of Microbiology Department, Leicester University, by the method of Maniatis et al., 1982, [p260]). In this protocol E.coli

lysogens BHB2690 (Dam) and BHB2688 (Eam) were grown separately, induced, mixed, concentrated and frozen in aliquots. The DNA was mixed in well by gentle stirring and incubated for 1 hour at room temperature. After this time the volume was made up to 700 µl with SM buffer (0.1 M NaCl, 0.01 M $MgSO_4$, 0.05 M Tris-HCl (pH 7.5), 0.01% (w/v) gelatin) and sterilised by adding 50 µl of chloroform. Any phage debris was removed by centrifuging for 5 minutes in a microfuge. 100 µl of this supernatant was used to infect 200 µl of plating cells and incubated at room temperature for 15 to 20 minutes before the newly acquired DNA was allowed to be expressed for 1 hour in 0.5 ml of nutrient broth. The whole infection was then plated on L-agar agar plates containing ampicillin at 100 $\mu g.ml^{-1}$.

2.8 Oligolabelling of DNA for hybridizations.

This method is based on that first described by Feinberg and Vogelstein (1984). The restriction fragment to be used as a probe was purified on a 0.6% low gelling temperature agarose gel (Seakem) made in TE buffer (see Section 2.5.3). The restricted DNA was loaded on to the gel such that the band of interest would contain approximately 0.5 µg of DNA. This band was visualized on a UV transilluminator and excised from the gel using a scalpel. All excess agarose was removed and the volume of the gel slice determined by weight (1 mg = 1 µl). The slice was placed in a screw capped eppendorf tube and water was added in the ratio of 1.5 ml water to 1 ml of gel. This was placed in a boiling water bath for 7 minutes to melt the agarose and denature the DNA. Samples to be used immediately were incubated at 37°C for 10 minutes. The remainder was frozen at -20°C and reboiled for 3 minutes, followed by 10 minutes at 37°C, before use.

The labelling reaction was carried out at room temperature by adding the following reagents in the order stated; distilled water (to a total

volume of 15 µl), 3 µl oligolabelling buffer, 0.6 µl BSA (10 mg.ml^{-1} enzyme grade, BRL), DNA (5 to 25 ng), 1 µl ^{32}P-dCTP and 0.6 µl of Klenow fragment (1 unit.µl^{-1}) (Pharmacia). Oligolabelling buffer consisted of solutions A, B and C mixed in the ratio of 2 : 5 : 3 and was stored at -20°C. Solution A consisted of 1.2 M Tris-HCl (pH 8.0), 120 mM $MgCl_2$, 1.7% (v/v) β-mercaptoethanol, 0.5 mM dATP, dTTP and dGTP (Sigma) (each triphosphate supplied as a 0.1 M stock dissolved in 3 mM Tris-HCl, 0.2 mM EDTA, pH7). Solution B was 2 M HEPES, titrated to pH 6.6 with NaOH. Solution C contained hexadeoxyribonucleotides (Pharmacia) evenly suspended in 3 mM Tris-HCl (pH 7.0), 0.2 mM EDTA, at 90 OD units per ml.

The labelling reaction was allowed to proceed for a minimum of 3 hours, but could be left over night. After this, 85 µl of stop solution were added (20 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.25% (w/v) SDS) and incorporation checked by taking 1 µl of the mixture diluted in 10 µl and drying 5 µl on to each of two Whatman nitrocellulose discs (25 mm, 0.45 µm pore size). One of these was washed in ice cold 5% (w/v) TCA solution for 3 minutes then rinsed in distilled water. The radioactivity on each of the dried discs was measured on the ^{3}H-channel of a Packard 3255 liquid scintillation counter.

2.9 In situ colony hybridization.

This method is based on that of Grunstein and Hogness (1975). The colonies to be tested were inoculated using sterile toothpicks in an ordered array in duplicate on to a gridded nitrocellulose filter (Schleicher and Schull, 0.45 µm). The filter was placed on a L-agar plate and colonies were allowed to develop over night at the permissive temperature for the strain. One of these filters was kept sealed in a petri dish as a master copy, the other was treated by laying the filter,

with the colonies uppermost, on a wick of 1MM Whatman filter paper which was soaked in a series of solutions at room temperature. The filter was first incubated for 10 minutes in 1.5 M NaCl, 0.1 M NaOH. This was followed by two incubations with 1.0 M Tris-HCl (pH 7.4) for 2 minutes, and finally 4 minutes in 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.4). Excess fluid was removed by blotting on a piece of dry 1MM Whatman paper before the filter was baked over night at 80°C.

The hybridization with ^{32}P-oligolabelled probe DNA (see Section 2.8) was performed at 65°C in a shaking water bath. The filter was washed in 3x SSC (65°C) for 15 minutes and then transferred to prehybridization mix (65°C) for 1 hour prior to addition of the probe DNA (20x SSC = 3.0 M NaCl, 0.3 M sodium citrate). The hybridization reaction was incubated over night with gentle agitation. Prehybridization mix consisted of 450 mM NaCl, 3 mM NaH_2PO_4 (pH 7.4), 3 mM EDTA, 5 $gm.l^{-1}$ Marvel powder (Cadburys), 1% SDS, 6% PEG 6000.

Next day the excess labelled DNA was removed by washing the filter in several changes of 1x SSC, 0.1% SDS (65°C) until the washings no longer contained any detectable amounts of radioactivity. After this a final stringency wash of 15 minutes was performed in 0.01x SSC containing 0.001% SDS (65°C). Finally the filter was rinsed in cold 3x SSC and air dried on 1MM Whatman filter paper. When dry, the filters were mounted on card and covered with cling film before autoradiographing using Kodak X-Omat S (1596) X-Ray film.

2.10 Southern Blot Analysis.

2.10.1 Acid/Alkali denaturation of gels.

Denaturation of the DNA in gels to be blotted with acid, prior to treating with alkali, has been shown to aid the transfer of large DNA fragments (Wahl *et al.*, 1979). Alkali denaturation is as described by

Southern (1980).

The DNA to be probed was restricted and run out on an agarose gel of appropriate percentage agarose concentration to achieve the best separation of the restriction fragments of interest. The gel was photographed alongside a ruler to give an indication of scale.

To denature the DNA the gel was twice washed in 0.25 M HCl for 15 minutes, followed by 2 washings in 0.5 M NaOH, 1 M NaCl for 15 minutes with occasional agitation. The gel was then briefly rinsed in distilled water before washing twice in 0.5 M Tris-HCl (pH 7.5), 3 M NaCl for 15 minutes. Finally the gel was soaked in 20x SSC solution for 20 minutes prior to transferring to the blotting apparatus.

2.10.2 Blotting Procedures.

The procedure used to transfer DNA restriction fragments to a membrane is based on the method of Southern (1975). The denatured gel was placed on a wick, held above a reservoir of 20x SSC. The wick was constructed from a sheet of 3mm Whatman filter paper with a sheet of cling film on top. A hole, slightly smaller than the gel, was cut in the cling-film to allow fluid to pass up through the gel. All air bubbles were removed from under the gel, then the parts of this that were not to be blotted were sceened off with double thicknesses of cling- film. A piece of Hybond N membrane (Amersham) soaked in 3x SSC was cut exactly to fit over the exposed section of gel. This was placed on top, followed by 4 pieces of 3MM Whatman paper cut to the same size. The first of these only was soaked in 3x SSC solution. Above this a wad of paper towels (~3 inches in height) and a glass plate were held in place by light weight. Wet paper towels were removed every 20 minutes for 2 hours before leaving the transfer to proceed over night at 4°C. The next day, the gel was viewed under UV light to check that transfer had occurred and then the filter was baked for at least 3 hours (or over

night) at 80°C. Hybridization and autoradiography were as described in Section 2.9, except the initial prehybridization wash in 3x SSC was omitted.

2.10.3 Removal of the probe DNA from hybond membrane for re-use.

This method is described in the Amersham handbook (Membrane transfer and detection methods, 1985). The labelled filter was soaked in a solution of 0.4N NaOH for 30 minutes at 45°C. This was followed by washing in 0.1x SSC, 0.1% (v/v) SDS, 0.2 M Tris-HCl (pH 7.5) for 30 minutes at 45°C. The filter was then air dried and autoradiographed to check that all the label had been removed. If the filter was heavily labelled, the initial washing in NaOH could be extended. The filter was then immediately ready for re-use.

2.11 Preparation of crude cell extracts. (Wilkins et al. 1981).

Total protein was prepared from 3 ml of an over night culture of bacteria, grown with appropriate antibiotic selection for any plasmids present. The cells were pelleted in universal tubes (Sterilin) in a bench top MSE centrifuge at top speed for 10 minutes. The supernatant was removed and the cells resuspended in 2.5 ml of 20 mM Tris-HCl (pH 7.6), 50 mM NaCl. The A_{450} of the sample was determined by measuring the absorbance given by a 1 in 20 dilution of the sample in a Gilford spectrophotometer. An appropriate volume of the bacterial suspension was then taken that contained the equivalent of 10 A_{450} units of cells and put into an eppendorf tube. The cells were again pelleted in as MSE microfuge for 5 minutes and resuspended in 150 µl of cracking buffer (62m5 mM Tris-HCl (pH 6.8), 3% (w/v) SDS, 10 % (v/v) glycerol, 0.05% (v/v) β-mercaptoethanol) and an equal volume of phosphate buffer. This was incubated at 60°C for 5 minutes, then in a boiling

water bath for 5 to 10 minutes, until the sample cleared. The lysate was then transferred to a Sorvall 10 ml polycarbonate tube and centrifuged in a Beckman 50Ti rotor at 30K for 30 minutes in a Beckman L5-65 centrifuge. The supernatant was transferred to an eppendorf tube and 1 µl of bromophenol blue solution added (2% (w/v) bromophenol blue in 50% ethanol, 50% acetic acid) to each sample. Approximately 20 µl of sample were run on a 10% SDS PAGE gel through a 7% stacking gel, at 4 mAmps for 4 to 5 hours until the dye front reached the bottom of the gel.

2.12 SDS-Polyacrylamide gel eletrophoresis.

The procedure used was based on that of Laemmli (1970), using a Raven vertical gel kit (IN/96). The gels were 1 mm in thickness and consisted of a 10% polyacrylamide plug gel, a separating gel of appropriate percentage to give good separation of the polypeptides of interest, and a 7% polyacrylamide stacking gel of a depth to provide a 1 cm separation between the bottom of the loading wells and the top of the separating gel. Buffer A (750 mM Tris-HCl (pH 8.8), 0.2% (w/v) SDS) was used to form the separating and plug gels and Buffer B (250 mM Tris-HCL (pH 6.8), 0.2% SDS) for the stacking gel. The composition of the gels is given in Table 2.12. The acrylamide solution was treated with a small amount of activated charcoal for 1 hour, filtered through two layers of 1MM Whatman filter paper, and stored in the dark at 4°C. The ammonium peroxydisulphate (Eastman Kodak Co., New York) solution (10 $mg.ml^{-1}$) was freshly prepared. TEMED (N,N,N',N'-tetramethylethylenediamide, Eastman Kodak Co.) was added immediately prior to pouring of the gel.

To avoid lateral spread of the samples during running, total protein prepared from 0.5 A_{600} units of cells was loaded in to the outside gel slots. Electrophoresis was carried out in running buffer (25 mM Tris-HCl (pH 8.3 - 8.6) 0.19% (w/v) SDS, 192 mM glycine) at 25 mA from a LKB 2197

power pack until the dye front entered the plug gel.

Polypeptides were visualised by staining in Coomassie blue stain (0.25% (w/v) coomassie blue (Gurr), 10% (v/v) glacial acetic acid, 25% (v/v) isopropanol), followed by washing gently in destain (10% (v/v) glacial acetic acid, 25% (v/v) propan-2-ol) for 2 to 3 hours to remove background staining.

2.13 Western Blotting.

The protein gel was trimmed to remove all unnecessary acrylamide. A piece of nitrocellulose (Schleicher and Schull, 0.45 μm pore size) was cut to exactly cover the gel slab and loaded into the electrophoresis tank between two sheets of Whatman 3MM filter paper and 2 Scotch Bright pads in a perforated perspex holder. Care was taken to remove all the air bubbles from between the gel and the nitrocellulose sheet to ensure good transfer. The tank was filled with transfer buffer (27 mM Tris, 350 mM glycine). Complete transfer was achieved after running for 8 hours at 50V at 4°C. To check efficient transfer had occurred, the gel was stained with Coomassie blue stain (see Section 2.12).

The nitrocellulose filter was washed for 10 hours in phoshate buffered saline (10 tablets [Sigma] dissolved in 1 litre distilled water) containg 0.05% (v/v) Tween (Sigma) (PBS-Tween) before incubating with the antibodies. In each case the antibody was diluted in PBS-Tween, the volume being kept to a minimum by sealing the filter with the antibody solution in a polythene sandwich bag. The bag was shaken vigorously for 1 to 2 hours at room temperature and after each incubation the filter was rinsed in several changes of PBS-Tween. A 1 in 100 dilution of primary antibody (rabbit anti-78 Kd *sog* polypeptide, Wilkins *et al.*, 1981) was used. The secondary antibody used was swine anti-rabbit IgG (Dakopatts: 1 in 50 dilution) and the tertiary was rabbit anti-horse radish

peroxidase (Dakopatts: 1 in 100 dilution). After a final wash in PBS-Tween the colour reaction was developed in a solution of PBS-Tween containing 5% (w/v) methanol, 0.6 $mg.ml^{-1}$ 4-chloro-1-naphthol. After about 2 minutes the reaction had reached completion and the filter was washed thoroughly in distilled water before drying in air.

CHAPTER THREE : Construction of a physical map of ColIb-P9 and characterisation of subclones of the plasmid

Introduction

Presented in this chapter is a summary of the restriction data now available for the plasmid ColIb-P9. The physical map is based on that of Uemura and Mizobuchi (1982a) (see Figure 1.4) which has been further refined, especially with respect to those unordered EcoRI fragments at coordinates 32.0 to 37.0 and 47.0 to 67.0. The map has been constructed using data gained from restriction analysis of specific regions of subcloned DNA from the parental plasmid. Isolation of these DNA fragments has sometimes relied on restriction sites created by transposon insertions, the construction of which is described in the next chapter.

For the sake of succinctness I shall not attempt to explain in detail the logic used in constructing these maps, but a brief description of some of the more relevant points is provided. For the rest, it is sufficient to say that the maps have been cross-checked by using several pairs of enzymes, ordering the fragments generated by these independently and then comparing the different maps for alignment. I have indicated where any ambiguities remain and have presented all the physical data which is of relevance. When referring to restriction fragments of EcoRI, HindIII and SalI, the fragments are named E, H and S respectively followed by a number. The latter corresponds to the relative size of the fragments when they are arranged in decreasing size, e.g. E5 is the fifth largest EcoRI fragment.

An investigation of the phenotypes conferred by the recombinant plasmids on their host strains was also performed. Once a detailed restriction map of these subcloned regions of DNA had been prepared, it was possible to superimpose on them the position of the genes encoded on them.

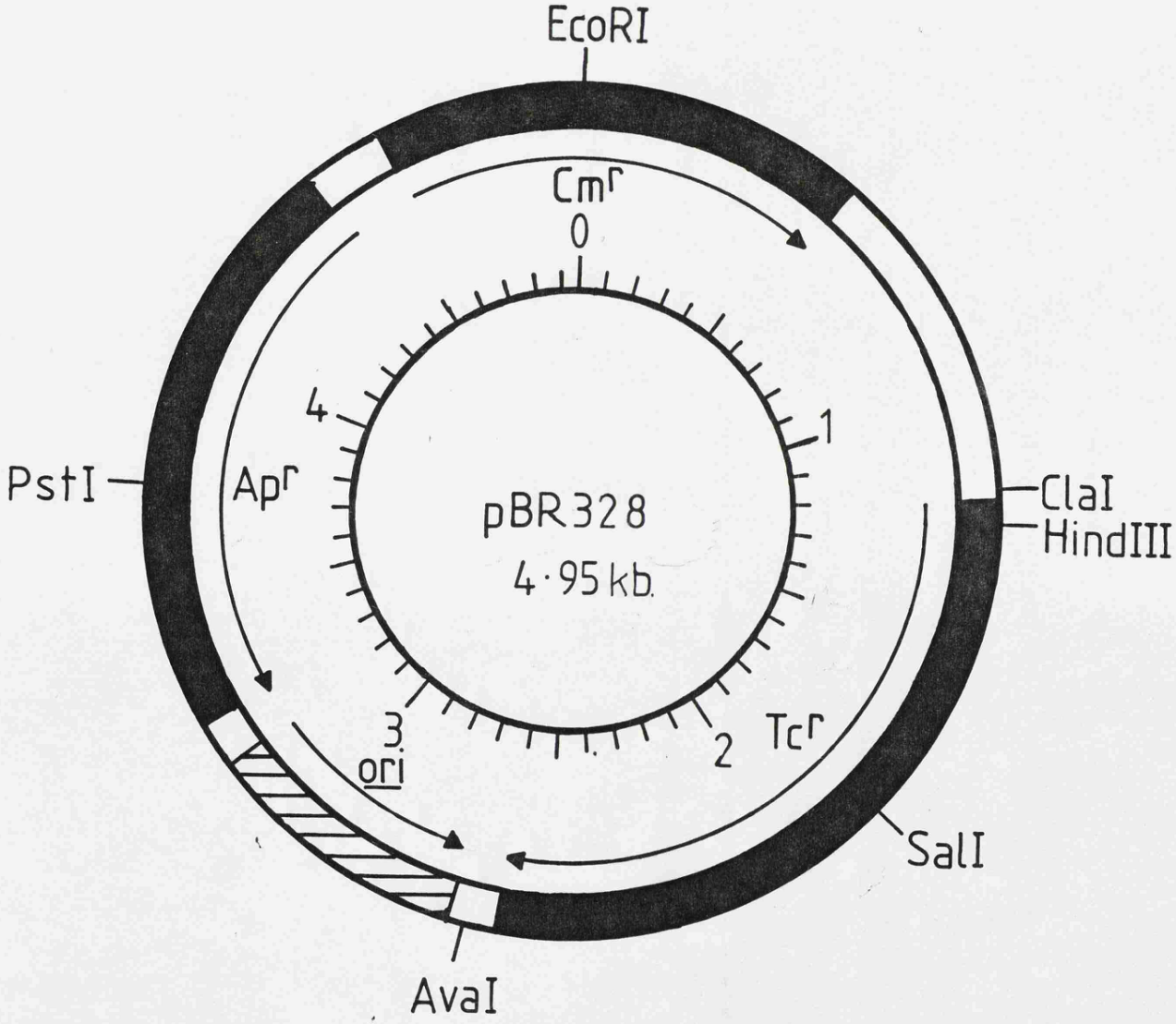
3.1 Subcloning of S3 and the location of oriT

A bank of recombinant plasmids containing cloned SalI fragments of ColIb-drd1 was generated using the cloning vector pBR328 (Figure 3.1.1). This was achieved by cutting ColIb DNA with this enzyme and then ligating the fragments generated in the presence of vector DNA linearised with the same enzyme. The ligation mixture was used to transform BW85 and transformants were selected on ampicillin and chloramphenicol. These colonies were tested for sensitivity to tetracycline to indicate the disruption of the tetracycline-resistance gene of the vector. Plasmid DNA was prepared and the size of the fragment inserted in to the vector molecule was determined and compared to the sizes of the SalI restriction fragments of ColIb. One such clone contained a SalI fragment of approximately 16 kb, which was equivalent to fragment S3 of ColIb. Further restriction analysis showed that this plasmid (pCRS3) contained EcoRI restriction fragments that corresponded to those unordered fragments contained within S3 in the map of Uemura and Mizobuchi (1982).

The order of these fragments was determined using double digests of the enzymes ClaI, BglII, XhoI, SstI and PstI (data presented in Tables 3.1.1 and 3.1.2). The EcoRI + SstI digest showed that the largest EcoRI fragment (corresponding to part of the E1 fragment of ColIb) was at one end of the cloned DNA, whilst the EcoRI + SalI digest indicated that part of E9 was at the extreme right hand side, attached to the vector DNA (see Figure 3.1.2). The SalI + PstI digest showed that the largest PstI fragment overlapped the region containing E9 DNA. Of the smallest EcoRI fragments, only the band corresponding to E15 of ColIb was not cut by PstI and therefore this must be adjacent to E9. The remaining two unordered EcoRI fragments lie between coordinates 3.8 and 7.0. Of these, only the 2.15 kb E14 band is cut by ClaI at coordinate 15.15 and therefore must be to the left of E15 in this region. In agreement with this, both E14 and

FIGURE 3.1.1 : Map of pBR328 showing relevant restriction sites.

The data is based on that given by Soberon et al., 1980. The solid regions of the circle indicate the extent of the coding region of the resistance determinants (Ap^r = ampicillin-resistance, Cm^r = chloramphenicol-resistance, Tc^r = tetracycline-resistance). The arrows indicate the direction of transcription of these genes. The origin of replication (ori) is shown by the hatched segment of the circle.


EcoRI
Cmr
0
1
2
3
4
pBR328
4·95 kb.
Apr
Tcr
ori
PstI
ClaI
HindIII
SalI
AvaI

Agarose gel showing restriction digests of pCRS3.

Lane	DNA	Enzyme
1	ColIbdrd-1	EcoRI
2	pCRS3	EcoRI
3	pCRS3	XhoI
4	pCRS3	ClaI
5	pCRS3	PstI
6	pCRS3	SstI
7	pCRS3	SalI

The EcoRI fragments of pCRS3 are marked on the left hand side of the gel, indicating those fragments which correspond to ColIb EcoRI fragments (c.f. Lane 1). The gel was 0.75% (w/v) run at 20 V for 16 hours.

TABLE 3.1.1 : SIZES OF FRAGMENTS OF pCRS3 GENERATED IN SINGLE RESTRICTION DIGESTS.

Enzyme	Fragment Sizes (kb)					Total
EcoRI	12.05	3.85	2.15	1.85	1.05	20.95
XhoI	15.5	5.4				20.9
ClaI	9.65	6.55	4.7			20.9
PstI	7.38	4.62	2.24	2.05	1.55	
	0.9 (D)		0.7	(0.48)[a]		20.82
BglII	11.35	6.85	2.65			20.9
SstI	~20					~20
SalI	16.0	4.9				20.9

(D) = Double Band

a : Figures in brackets indicate sizes of fragments inferred from the physical map

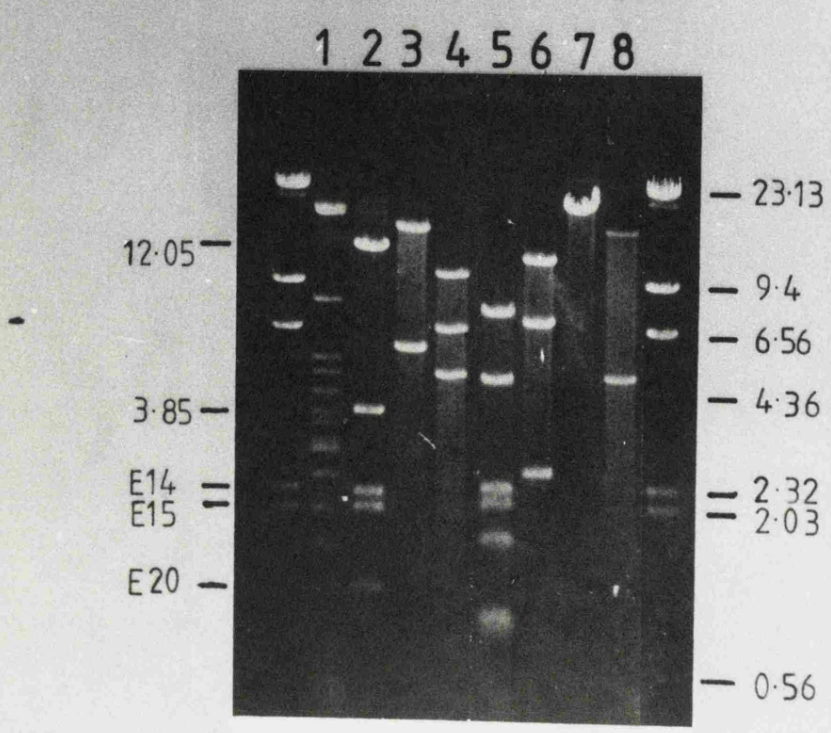


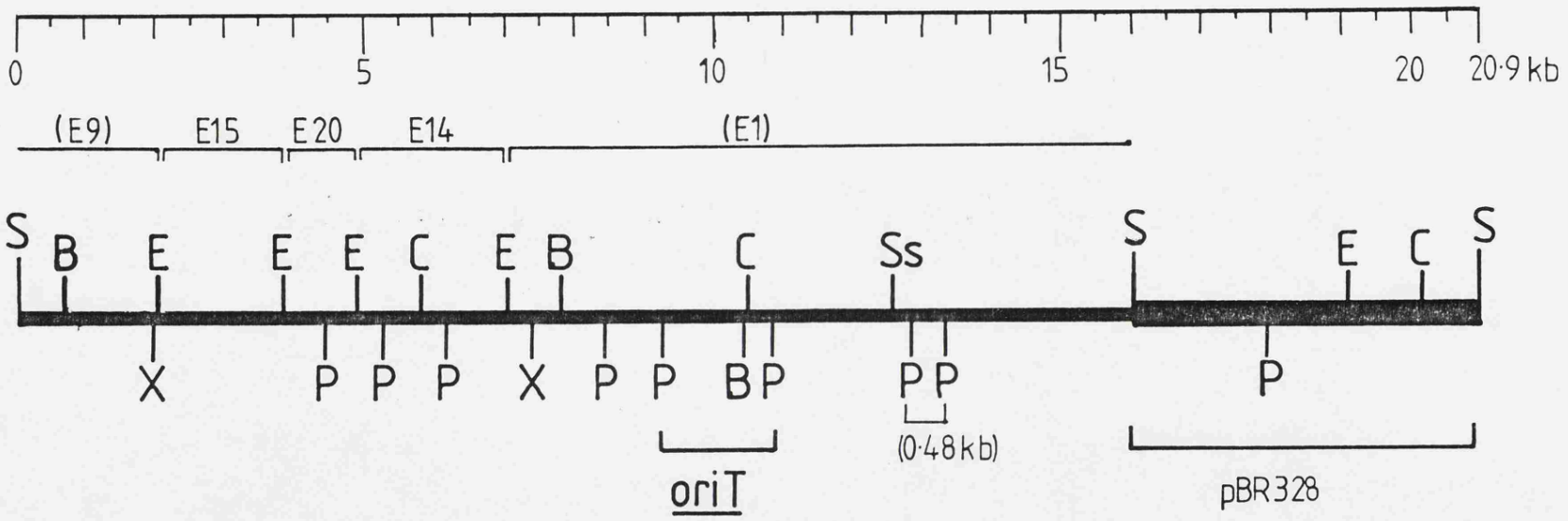
TABLE 3.1.2 : SIZES OF FRAGMENTS OF pCRS3 GENERATED IN DOUBLE RESTRICTION DIGESTS

Enzyme	Fragment Sizes (kb)					Total
SalI + EcoRI	9.0	3.05	2.15	1.93	1.85	
		1.05				20.88
SalI + XhoI	8.67	5.4	4.9	1.93		20.9
SalI + PstI	4.4	2.98	2.70	2.24	2.05	
	1.92	1.55	0.9(D)	(0.48)		20.82
SalI + SstI	12.55	4.9	3.45			20.9
SstI + BglII	9.05	6.85	2.65	2.35		20.9
SstI + ClaI	9.65	6.55	4.7			20.9
SstI + XhoI	10.28	5.4	5.15			20.83
SstI + PstI	7.38	4.62	2.24	1.78	1.55	
	0.9(D)	0.7	(0.48)	(0.37)		20.92
XhoI + ClaI	9.65	4.05	3.12	2.48	1.57	20.87
XhoI + BglII	11.35	5.4	2.65	1.05	(0.42)	20.87
XhoI + PstI	4.91	4.62	2.47	2.05	1.55	
	1.21	1.03	0.9(D)	0.7	(0.48)	20.82

(D) = Double Band

a : Figures in brackets indicate sizes of fragments inferred from the physical map

FIGURE 3.1.2 : Restriction map of pCRS3.



Restriction sites indicated are for BglII (B), ClaI (C), EcoRI (E), PstI (P), SalI (S), SstI (Ss) and XhoI (X). Above the map, the EcoRI fragments of ColIb are indicated with the numbers in brackets representing incomplete fragments. Also shown is the 1.55 kb PstI fragment thought to contain the oriT site and the location of the 0.48 kb PstI fragment, the presence of which was inferred from the restriction analysis.

E20 are cut by PstI whereas if they were arranged in the opposite orientation only E15 would be cut (possibly undetectably) at a site very close to one end of the fragment. The 0.48 kb PstI fragment was not seen on the mapping gel, but its presence is inferred because the PstI site at 13.27 kb is fixed and SstI cuts the 2.05 kb PstI band to give a new 1.78kb band and an undetected band of 0.37 kb. The remaining distance between these fixed sites is 0.48 kb and the existence of a band of this size has been confirmed by other workers in this laboratory (C. Wymbs, unpublished data). The final restriction map of pCRS3 is shown in Figure 3.1.2.

Location of oriT

The ColIb origin of transfer (oriT) had previously been cloned in this laboratory on a 1.55 kb PstI fragment which contained ClaI and BglII sites very close together. From the restriction map of pCRS3 it appears that this may correspond to the PstI fragment 5 (Table 3.1.1). To test whether pCRS3 carried the ColIb oriT, the plasmid was introduced by transformation into a recA strain containing the plasmid pCR9 (ColIbdrd-1 tra$^+$ E12::Tn5). The recA background reduces the likelihood of recombination between the parental plasmid and the subcloned ColIb DNA. The resultant strain was used as a donor in a mating with BW97. Recipient cells were selected for acquisition of either Km^R (pCR9) by conjugative transfer, or Ap^R (pCRS3) by mobilization. Frequency of mobilization is given as the number of recombinant plasmids transferred per transfer of the conjugative plasmid. The results (Table 3.1.3) show a mobilization frequency of 0.42 for pCRS3. By comparison, the frequency of mobilization of the plasmid containing the cloned 1.55 kb PstI fragment (pLG2009) is given as 0.84 (Merryweather et al. 1986a). The slightly lower frequency of mobilization of pCRS3 probably reflects its much larger size (20.9 kb as compared with 3.1 kb). Therefore it would seem that pCRS3 does contain the origin of transfer and that it is located in the 1.55 kb PstI fragment

TABLE 3.1.3 : MOBILIZATION OF pCRS3

Donor Strain	Transconjugants/ml Ap^R Nal^R (pCRS3)	Transconjugants/ml Km^R Nal^R (pCR9)	Mobilization[a] of pCRS3
J08(pCR9)	NIL	1.4×10^8	--
J08(pCRS3)	NIL	NIL	--
J08(pCR9,pCRS3)	6.8×10^7	1.6×10^8	0.42

a : Mobilization frequency is given as the number of mobilizable plasmids transferred per conjugative plasmid transferred.

Frequency of transfer of the plasmids was determined after a 1 hour mating at 37°C in liquid culture using BW97 as the recipient strain. Transfer of pCRS3 was measured by plating the mating mixture on L-agar plates containing Amp ($100 \mu g ml^{-1}$) and Nal ($50 \mu g ml^{-1}$) and transfer of pCR9 by plating on Km ($50 \mu g ml^{-1}$) and Nal ($50 \mu g ml^{-1}$). The vector component of pCRS3 (pBR328) is not mobilizable.

indicated in Figure 3.1.2.

As described in Chapter 1, the origin of transfer of F-like plasmids is found to lie just beyond the end of the long traY-Z operon and transfer proceeds such that the conjugation genes are transferred last during the mating process. The oriT sites of the IncP plasmids RP4 and RK2 are also found in an analogous position, therefore it would seem plausible that the ColIb transfer origin may also lie at the end of a block of transfer genes. On the assumption that some of these genes were contained within S3, this fragment of DNA was used to generate the set of Tn5 insertions in ColIb described in Chapter 4.

3.2 Subcloning of the E3 region and orientation of the plasmid primase gene.

The remaining part of ColIb that contained unordered EcoRI fragments in the map of Uemura and Mizobuchi (1982a) was that within S1. This fragment is some 40 kb and was too large to be easily subcloned for restriction analysis. Therefore to isolate the region containing all these EcoRI fragments, a new SalI site was created by insertion of Tn5 into E10 (see Chapter 4). The same shotgun cloning technique was used as described in Section 3.1, except that the pBR328-SalI generated recombinants were used to transform BW86. An insert of approximately 23 kb in the vector was identified by restriction analysis with SalI and was found to contain EcoRI restriction fragments corresponding to E3, E5, E16, E19 and E21 of ColIb. This plasmid was termed pCRS1a and ordering of the EcoRI fragments was achieved using the enzymes BglII, EcoRI, SalI and SphI. Restriction data and a map of pCRS1a are presented in Tables 3.2.1 and 3.2.2 and in Figure 3.2.1.

The SalI + EcoRI digest orientated the inserted DNA with respect to the vector and showed that 1.2 kb of ColIb E18 (either this fragment or

TABLE 3.2.1 : SIZES OF FRAGMENTS OF pCRS1a GENERATED IN SINGLE RESTRICTION DIGESTS.

Enzyme	Fragment Sizes (kb)					Total
SalI	~23	4.9				~27.9
EcoRI	8.4	6.3	5.0	4.25	1.90	
	1.07	0.97				27.89
BglII	14.25	5.1	4.55	4.05		27.95
SphI	8.55	7.88	5.75	5.6		27.78

TABLE 3.2.2 : SIZE OF FRAGMENTS OF pCRS1a GENERATED IN DOUBLE RESTRICTION DIGESTS.

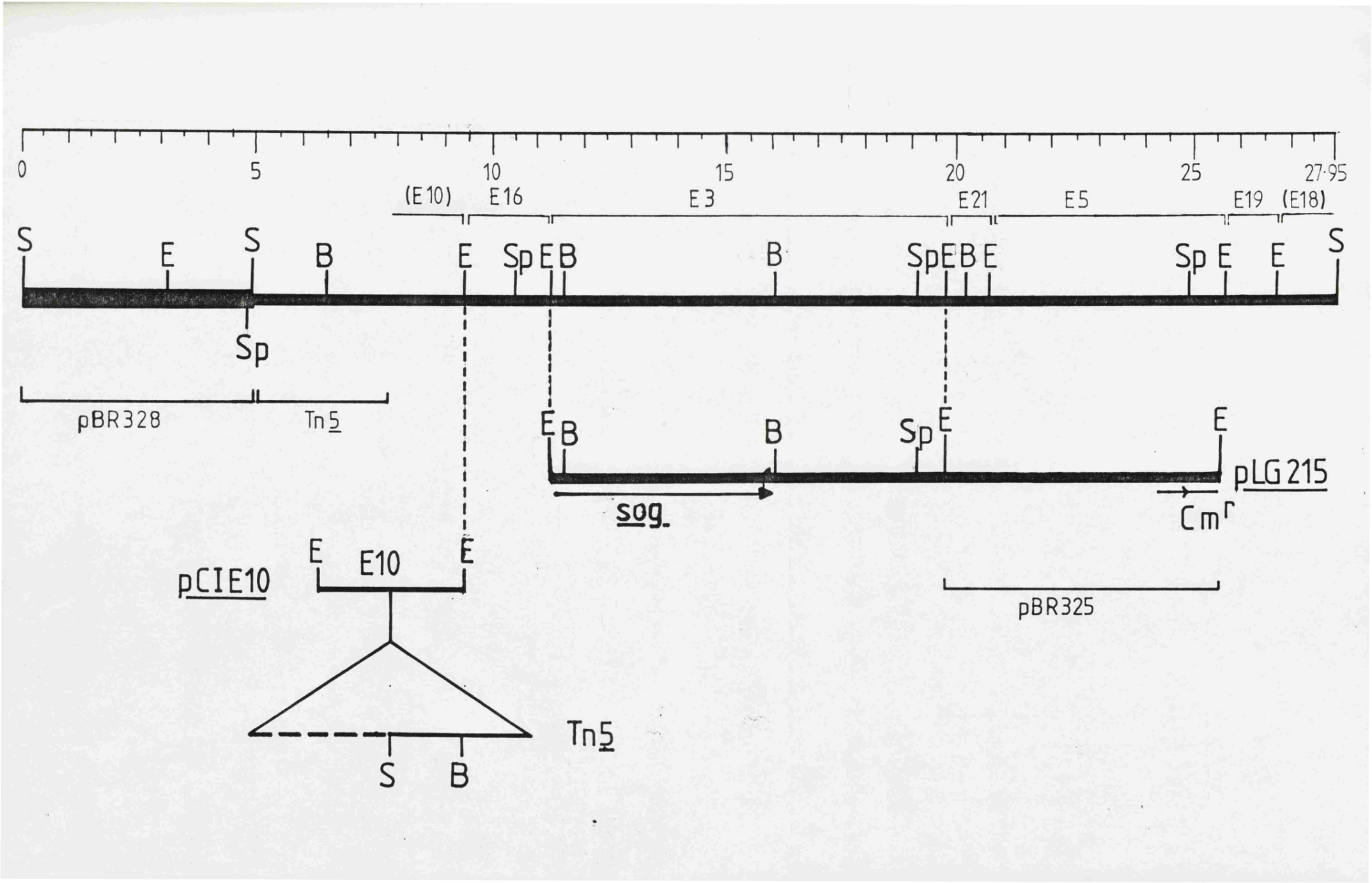
Enzymes	Fragment Sizes (kb)					Total
SalI + EcoRI	8.4	5.0	4.5	3.05	1.90	
	1.85	1.2	1.07	0.97		27.94
SalI + BglII	7.77	5.1	4.9	4.55	4.05	
	1.57					27.94
BglII + SphI	7.88	4.7	4.55	4.05	2.95	
	1.67	1.1	1.05			27.95
EcoRI + SphI	7.8	4.6	4.25	4.20	1.75	
	1.07(D)	0.97	0.8	(0.7)	(0.6)	27.81
EcoRI + BglII	5.0	4.55	4.25	3.40	3.0	
	1.90	1.07	(0.97)	(0.25)		27.89

a : Figures given in brackets are sizes of fragments inferred from the physical map.

(D) : Double Bands.

FIGURE 3.2.1 : Restriction map of pCRS1a.

Restriction sites indicated are for BglII (B), EcoRI (E), SalI (S) and SphI (Sp). Above the map, the EcoRI fragments of ColIb are indicated, with numbers in brackets representing incomplete fragments. Below the map, the plasmid pLG215 is aligned to show the arrangement of BglII and SphI sites in this fragment and the direction of transcription of the sog gene (indicated by the arrow) which is being expressed from the Cm^r gene promoter. Also shown is the site of Tn5 in the E10 fragment of pCIE10, from which pCRS1a was constructed. This shows how the SalI and BglII sites at coordinates 4.95 and 6.5 of pCRS1a originate from Tn5 (see Chapter 4 for a description of pCIE10).


0
5
10
15
20
25
27·95
(E10)
E16
E3
E21
E5
E19
(E18)
S
E
S
B
E
Sp
E
B
B
Sp
E
B
E
Sp
E
E
S
Sp
pBR328
Tn5
E
B
B
Sp
E
E
pLG215
sog
Cm r
pCIE10
E
E10
E
pBR325
Tn5
S
B

E19 was known to contain the SalI site from the Uemura and Mizobuchi map) was attached to the vector at one end and as expected, the transposon DNA and part of E10 was attached to the other end of the vector. (The distance from the end of the transposon to the EcoRI site bounding E10 was already known from the construction of the Tn5 insert, see Chapter 4.) Once the location of the Tn5 DNA was known, a SalI + BglII double digest allowed the largest BglII fragment to be positioned. From the map of Uemura and Mizobuchi, ColIb E18 was known to be adjacent to E19, with E5 adjacent to this. That these bands were not cut in a BglII + EcoRI double digest supports their positioning within the large BglII fragment. BglII + SphI digests located the SphI sites, along with E16 and E5 which are cut by SphI at positions 10.45 and 20.05 respectively and confirms the positioning of E5. Only E3 and E21 now remain to be ordered within pCRS1a. The E3 fragment had been previously cloned into pBR325 (to give plasmid pLG215) and restriction data indicated that a single SphI site existed close to one end (~0.6Kb) of the fragment (Figure 3.2.1; Merryweather, 1986). With this information, only one orientation of the two remaining fragments is possible to generate SphI bands that correspond to those seen for pCRS1a (see Figure 3.2.1.). This arrangement also positions the BglII sites internal to the E3 fragment in agreement with the map of pLG215.

Orientation of the sog gene.

The E3 fragment of ColIb carries the gene sog specifying the plasmid-encoded DNA primase which is coordinately expressed with the transfer genes. Although it had been located on this fragment and its direction of transcription from pLG215 determined by deletion studies (Boulnois et al., 1981), sufficient mapping data did not exist to orientate the gene with respect to the whole plasmid. From the map of pCRS1a this is now possible and it is seen that the gene is transcribed anticlockwise on the standard

map of ColIb, towards oriT (see Figures 3.2.1 and 3.4).

Expression of sog from pCRS1a.

The cloned sog gene in plasmid pLG215 was found previously to be transcribed not from its natural promoter but from the promoter of the Cm^R gene in the pBR325 vector (Boulnois et al., 1982). It was hoped that pCRS1a would contain the gene with its own promoter intact. When expressed, the plasmid primase can suppress the effect of the host dnaG3 temperature-sensitive (ts) mutation (Wilkins et al., 1981). To test whether pCRS1a expressed the sog primase, the plasmid was introduced into BW86 (a dnaG3ts strain) and the colony-forming ability of the strain determined at the non-permissive temperature (40°C). The results of this test are seen in Table 3.2.3, and show that pCRS1a alone does not express sog. The plasmid pLG250 is a Kan^R derivative of ColIbdrd-1 which has a point mutation in the sog gene and does not express primase activity (Chatfield et al., 1982). When pCRS1a was introduced in to BW86(pLG250), the sog gene was expressed to the same level as the Sog^+ control, pCR9.

Although pCRS1a did not express sog activity in isolation, this did not necessarily imply that the promoter was not present. As the sog gene is one of a number of coordinately expressed conjugation genes (Wilkins et al., 1981), it is possible that a positively acting factor is required in trans for the expression of the gene and that this is absent from pCRS3. The Sog^- plasmid pLG250 can supply this factor(s) and therefore in the presence of this plasmid, sog can be expressed from pCRS1a from its own promoter.

The strain used in this suppression test, was $recA^+$. To rule out the possibility that sog expression in BW86(pLG250,pCRS1a) was the result of a homologous recombination event between the two plasmids, plasmid DNA was prepared from the strain and used to retransform BW86. The resultant

TABLE 3.2.3 : AFFECT OF pCRS1a ON COLONY-FORMING ABILITY OF BW86 AT HIGH TEMPERATURE

Strain	c.f.u[a]/ml at 30°C	c.f.u/ml at 40°C	Colony-Forming Ability
BW86	3.8×10^7	0	$<2.6 \times 10^{-8}$
BW86(pCRS1a)	2.6×10^8	3.9×10^2	1.5×10^{-6}
Bw86(pCR9)	2.6×10^7	4.4×10^7	1.7
BW86(pLG250)	1.8×10^6	5.8×10^1	3.2×10^{-5}
BW86(pLG250,pCRS1a)	5.7×10^6	8.2×10^6	1.4
BW86(pCRS1a)[b]	2.4×10^7	0	$<4.2 \times 10^{-8}$

a : c.f.u = colony-forming units.

b : This strain was constructed with DNA obtained from the strain BW86(pLG250,pCRS1a)

Expression of the sog gene was determined by plating cells on non-selective L-agar to give single colonies at both the permissive (30°C) and non-permissive temperatures (40°C) for the dnaG3 lesion in BW86. Rescue of colony-forming ability at 40°C is seen as a measure of plasmid primase activity and is expressed as the ratio of colonies formed at 40°C to the number formed at 30°C (colony-forming ability).

Amp^R, Cm^R, Km^S transformants did not suppress the dnaG3 mutation at 40°C and contained a plasmid with a restriction pattern identical to that of pCRS1a. This rules out the possibility that the expression of sog in the presence of pLG250 was from a Sog^+ recombinant of pLG250 and indicates that expression from the sog promoter requires a trans-acting factor from ColIb. This is the first direct evidence that ColIb controls the coordinate expression of its conjugation genes via a trans-acting factor, similar to the way that the F sex factor controls expression of the traY-Z operon by traJ .

In summary, both the location and direction of transcription of the sog gene from ColIb are now known. The promoter may lie anywhere in the 3.5 kb of DNA between the site of the Tn5 insertion in E10 and the left end of the E3 fragment as drawn. It has already been deduced from Tn5 insertion analysis that the sog gene either lies at the end of a transfer operon, or that putative genes which are downstream of sog in the same transcriptional unit have no effect on the efficiency of conjugative transfer (Merryweather et al., 1986b). Because the promoter for the sog gene could lie anywhere in a 3.5 kb region, the possibility is not precluded that tra genes may lie upstream of sog in the same transcriptional unit. This remains an area for future study.

3.3 Cloning of S5 and location of the origin of replication and incompatibility determinant of ColIb.P9.

A report in Walia and Duckworth (1984) suggested that the region essential for replication of ColIb (rep) was present on S5 and that the incompatibility determinant (inc) was also located in this region. This positioning was supported by evidence from Furuichi et al., (1984) that S7, when fused to the part of the rep region of the plasmid R64 could recreate a functional origin. Some preliminary data from transposon

FIGURE 3.3.1 : Restriction map of pCRS5.

Restriction sites indicated are for AvaI (A), BglII (B), PstI (P) and SalI (S). Above the map, the 2.10 kb. 2.35 kb and 5.55 kb PstI fragments referred to in the text are indicated. Also shown is the probable location of the origin of replication (oriV) and the incompatabiltiy determinant (Inc) and the the chloramphenicol-resistance (Cm^r) determinant, ampicillin-resistance (Ap^R) determinant and the origin of replication (ori) of pBR328 .

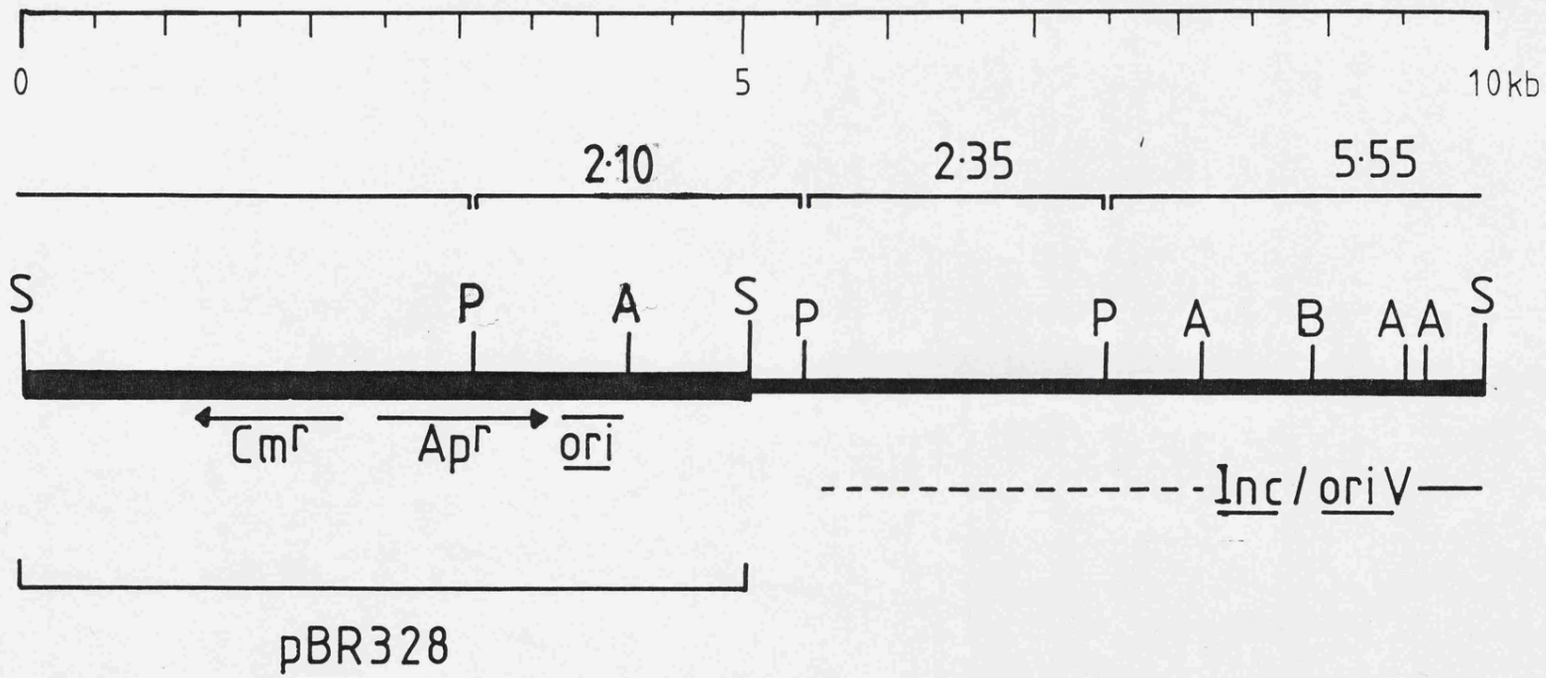

0
5
10kb
2·10
2·35
5·55
S
P
A
S
P
P
A
B
A
A
S
Cmr
Apr
ori
Inc / ori V
pBR328

TABLE 3.3.1 : SIZE OF FRAGMENTS OF pCRS5 GENERATED FROM SINGLE RESTRICTION DIGESTS.

Enzyme	Fragment Sizes (kb)				Total
SalI	5.0	4.95			9.95
AvaI	4.55	3.9	1.43	(0.12)	10.0
PstI	5.55	2.35	2.10		10.0
BglII	~10				10

a : Figures given in brackets are sizes of fragments inferred from the physical map.

Agarose gel showing restriction digests of pCRS5.

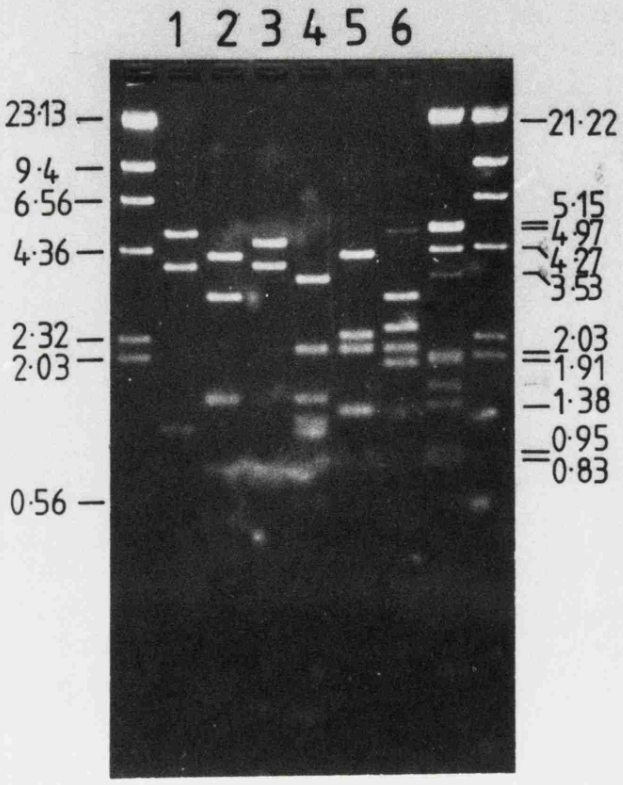
Lane	DNA	Enzymes
1	pCRS5	SalI + BglII
2	pCRS5	SalI + AvaI
3	pCRS5	BglII + AvaI
4	pCRS5	PstI + AvaI
5	pCRS5	PstI + BglII
6	pCRS5	PstI + SalI

Molecular weight standards are λ x HindIII and λ x HindIII + EcoRI. The gel was 0.8% (w/v), run at 25 V for 16 hours.

TABLE 3.3.2 : SIZE OF FRAGMENTS OF pCRS5 GENERATED FROM DOUBLE RESTRICTION DIGESTS.

Enzymes	Fragment Sizes (kb)					Total
SalI + BglII	4.95	3.9	1.12			9.97
SalI + AvaI	4.15	3.10	1.43	0.8	0.4	
	(0.12)					10.0
BglII + AvaI	4.55	3.9	0.76	0.64	(0.12)	10.0
PstI + AvaI	3.5	2.10	1.43	1.22	1.05	
	0.47	(0.12)				9.89
PstI + BglII	4.15	2.35	2.10	1.40		10.0
PstI + SalI	3.1	2.5	2.10	1.93	0.42	9.95

a : Figures given in brackets indicate sizes of fragments inferred from the physical map.



mutagenesis of ColIb*drd-1* (Chapter 4) indicated that the left end of fragment S5, as drawn, has some role in conjugation. Therefore it was decided to investigate the phenotype of this plasmid in two ways: firstly, to see if the cloned DNA contains a functional *rep* region and secondly, to determine where this function lies within S5, i.e. to see whether it overlaps with the region involved in conjugation or if the two regions are separate.

Mapping ofpCRS5.

From the bank of *SalI* recombinant plasmids generated when isolating pCRS3 (Section 3.1), one was isolated that carried a 5.0 kb insert. This corresponded to fragment S5 of ColIb and the plasmid was termed pCRS5. A map of this is presented in Figure 3.3.1 along with relevant restriction data in Tables 3.3.1 and 3.3.2. The mapping of this plasmid was very straightforward, being made easier by the presence of the single *BglII* site in the cloned ColIb DNA. This allowed the position of the 1.43 kb *AvaII* and 5.5 kb *PstI* fragments to be identified and the double digests with *SalI* and the various other enzymes located the remainder of the fragments. An ambiguity arises in the *BglII* + *AvaI* and *SalI* + *AvaI* digests, suggesting that a very small (0.12 kb) *AvaI* band exists between coordinates 9.5 - 9.62. This band was subsequently detected on a 2% overloaded agarose gel (data not shown).

Evidence for the presence of an incompatibility determinant.

Initially it was established that pCRS5 contained an *inc* region by transforming a strain carrying the ColIb derivative pCR9 with pCRS5 DNA. When this was done, either Km^R or Ap^R transformants could be obtained, but selecting for both resistances together produced only two transformant colonies. After purification these were shown to be only Km^R resistant (Table 3.3.3). Subsequent testing of the colonies selected for single

TABLE 3.3.3 : INCOMPATIBILITY OF pCR9 AND pCRS5.

Antibiotic in plates	Transformants/ml + pCRS5 DNA	Colony forming units/ml
Ap	6.1×10^3	NIL
Km	8.2×10^7	7.8×10^7
Ap + Km	2[a]	NIL

a : When streaked to single cells these transformants were found to be resistant only to Km.

Competent cells of J08(pCR9) were transformed with 0.5 μg of pCRS5 DNA. After newly acquired DNA had been allowed to express for 1 hour at 37°C, the cells were plated out to give single colonies on L-agar plates containing either Amp ($100 \mu g ml^{-1}$) or Km ($50 \mu g ml^{-1}$) or on plates containing both of these, and allowed to grow overnight. As a control, non-transformed cells were also plated out on the same selective plates.

antibiotic resistances showed that they did not retain the other resistance marker. Isolation of Amp^R transformants indicated that transformation had occurred efficiently, whilst the number of kanamycin resistant colonies showed that the competent cells still retained pCR9 at the time of plating out. The loss of one of the plasmids in each case suggests that pCRS5 cannot coexist with pCR9 and that it must therefore carry an incompatibility determinant.

Evidence for the presence of a functional oriV.

To test for a functional ColIb origin of replication (oriV), a strain with a temperature-sensitive polA mutation (JC411polA214) was used. The vector used to clone S5, pBR328, is based on pMB1 which is a ColE1-like replicon (Soberon et al., 1980). This type of replication origin requires host DNA polymerase I activity encoded by the chromosomal polA gene for replication (see Kingsbury and Helinski, 1973). Thus in the absence of a polA-independent origin cloned in to the vector, pBR328 is rapidly lost from JC411polA214 at the non-permissive temperature (Durkacz and Sherratt, 1973).

The plasmids pCRS5 and pBR328 were introduced into the strains JC411 and JC411polA by transformation at the permissive temperature, 30°C. These strains were grown for one mass doubling (from A_{600} of 0.05 to 0.1) in the presence of ampicillin. At this point samples were taken and plated on L-agar with or without ampicillin to determine what proportion of cells carried each of the plasmids. The remainder of the cells were resuspended in nutrient broth prewarmed to either 30°C or 40°C, to give approximately 5×10^4 cells per ml. The cells were then grown for about ten mass doublings to an A_{600} of 0.1. At this time the proportion of plasmid-carrying cells was determined by plating on selective and non-selective media (Table 3.3.4).

From the results of this test it can be seen that both plasmids were

After growing the cells in the presence of ampicillin (50$\mu g ml^{-1}$), the selection for the plasmid was removed by resuspending the cells in prewarmed nutrient broth to give 5 x 10^4 cells per ml. The cells were then grown for ten generations at 30°C and 40°C. The percentage of plasmid carrying cells was determined at each stage by spreading the cells out to give single colonies on L-agar plates, plus and minus ampicillin (100 $\mu g ml^{-1}$).

TABLE 3.3.4 : TEST FOR A FUNCTIONAL ColIb ORIGIN ON pCRS5.

	Strain	c.f.u./ml	c.f.u./ml (Ap^R)	Plasmid carrying cells (%)
T = 0	JC411(pBR328)	3.4×10^7	2.2×10^7	66
	JC411*polA*(pBR328)	1.2×10^7	4.8×10^6	40
	JC411(pCRS5)	6.1×10^7	3.7×10^7	61
	JC411*polA*(pCRS5)	7.3×10^7	5.3×10^7	73
T = 10	JC411(pBR328)	1.4×10^7	1.2×10^7	86
30°C	JC411*polA*(pBR328)	8.9×10^6	1.0×10^4	0.12[a]
	JC411(pCRS5)	2.9×10^7	1.8×10^7	62
	JC411*polA*(pCRS5)	6.0×10^7	3.8×10^7	62
T = 10	JC411(pBR328)	2.9×10^7	2.0×10^7	68
40°C	JC411*polA*(pBR328)	5.3×10^{6}[b]	0	$<1.9 \times 10^{-7}$
	JC411(pCRS5)	2.7×10^7	1.3×10^7	48
	JC411*polA*(pCRS5)	1.7×10^7	4.3×10^6	25

a : Loss of pBR328 at the permissive temperature when selection for the plasmid is not maintained suggests that the *polA* phenotype may be partially expressed even at the permissive temperature.

b : The number of viable cells is probably reduced because cells carrying ColE1 replicons filament extensively when replication is inhibited. (Durkacz and Sherratt, 1973)

T = the number of generations of growth without selection.

stable in the parental JC411 strain at all temperatures. Some plasmid loss was seen for pCRS5 at high temperature (68% dropped to 48%), but this is probably a consequence of the rapid growth rate of the cells. In the polA strain however, growth at high temperature produces a complete loss of pBR325 whereas pCRS5 is still present in 25% of cells. This partial stabilization of the ColE1 replicon indicates that at least part of the ColIb oriV must be present on S5. Furuichi et al. (1984) reported that part of the rep region of ColIb was located on the left hand end of S7, the fragment adjacent to, and to the right of S5 on the standard map. Taken with the results obtained for S5, it would seem that the origin of ColIb spans the SalI site at the border of fragments S5 and S7, and that the Inc determinant is located to the left of oriV on S5 close to this region. By analogy with other low copy number origins, both these regions may contain several tandem repeat sequences as part of their structure. As described in Chapter 1, deletion of some but not all of these repeats reduces the stability of the origin. Therefore the partial stabilization of pBR328 by S5 may reflect the fact that some structural features of the origin are to be found on S7 and that they are required for a fully functional ColIb origin of replication.

Location of rep and inc determinants on S5.

As already mentioned, transposon insertions into S5 had a Tra$^-$ phenotype (Chapter 4). These insertions were clustered towards the left hand side of the fragment adjacent to S1, and had no apparent effect on either the stability or incompatibility of the plasmids. This correlated well with the suggestion that inc and oriV were located on the right hand end of the fragment adjacent to S7.

To try and delimit the region of S5 involved in these functions, the PstI sites at coordinates 3.10, 5.37 and 7.47 on the map of S5 were used to subclone the Inc determinant. By cutting the plasmid with this enzyme

TABLE 3.3.5 : INCOMPATIBILITY OF DELETED pCRS5 PLASMIDS.

Plasmid in Recipient	Cm^R Transconjugants/ml (pCRS5 derivative)	Km^R Transconjugants/ml (pLG221)	Transconjugants/ml (Cm + Km)
pCRS5 ΔPst1-1	5.7×10^7	6.0×10^6	20[a]
pCRS5 ΔPst1-2	7.3×10^7	7.1×10^5	0

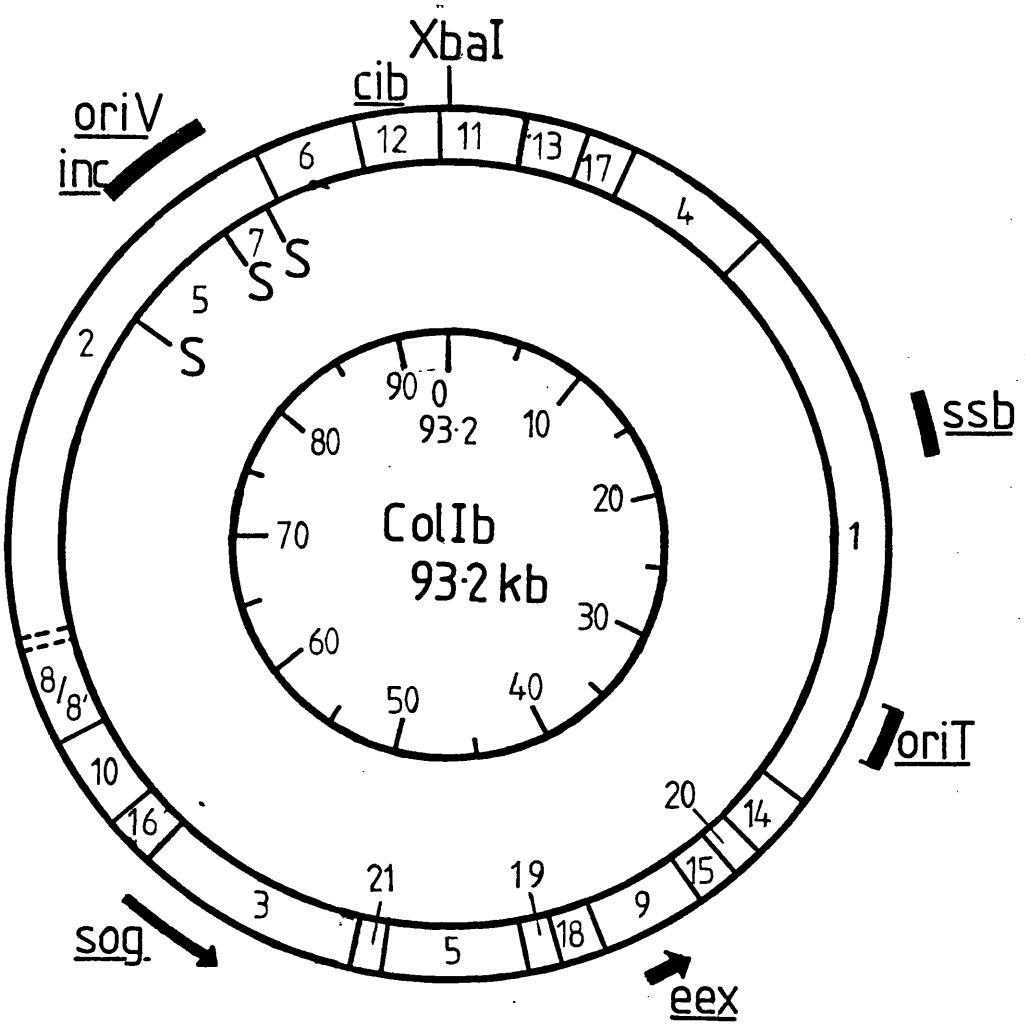
a : After purification to single colonies, all these doubly resistant transconjugants were found to have only single resistances to either Cm or Km.

The plasmids pCRS5 ΔPstI are the deletion derivatives of pCRS5 (see text). Incompatibility was determined from the antibiotic resistance pattern of transconjugants when BW85 carrying the deleted pCRS5 plasmids were used as recipients in a 1 hour liquid mating at 37°C, with W3110(pLG221) as a donor strain. Donor cells were killed by Sm (25 $\mu g.ml^{-1}$).

and then religating the mixture, it was possible to isolate in Cm^R Ap^S transformants of BW85 which contained plasmids that had lost the internal 2.10 kb PstI fragment. Deleting this fragment removes the Ap^R determinant but also removes the pBR328 origin of replication. That viable plasmids could be constructed in the absence of this fragment provides more evidence for the presence of a functional origin of replication on S5 as presumably the deleted plasmids must be utilizing it. The resultant plasmids were analysed by restriction with PstI and were found to contain both the 5.55 kb fragment, carrying the chloramphenicol- resistance determinant from the vector, and the 2.35 kb PstI fragment internal to the S5 DNA (see Figure 3.3.1). Two of these plasmids were tested for incompatibility with pLGL221, by using strains containing them as recipients in a standard liquid mating with the donor strain W3110(pLG221). The mating mixture was plated out after 1 hour on L-agar plates containing Sm and either Km or Cm or both of these antibiotics. The results (Table 3.3.5) demonstrate that at the time of plating out, most cells still retained the subcloned regions of S5 and that most had also received the ColIb plasmid. However, only twenty transconjugants were isolated that contained both plasmids, and after purification these were found to have segregated into strains with only a single resistance to either Km or Cm. Therefore these deleted plasmids still retain the inc determinant found in pCRS5.

Deletion of the 2.10 kb PstI fragment also removes 0.4 kb of the left end of the S5 DNA. Most origins so far characterised are contained within 1.0 - 0.5 kb of DNA. (see Chapter 1). Knowing that the ColIb origin spans one of the SalI sites of pCRS5, deletion of 0.4 kb would be expected to remove most of the origin region, especially as the size of the origin is already thought to be reduced by removal of sequences in S7. Since the PstI deletions of pCRS5 still retain a functional oriV, it is probable that it lies to the right end of the fragment as drawn. The presence of the

FIGURE 3.4 : EcoRI restriction map of ColIb.



A revised Uemura and Mizobuchi map including the mapping data from Sections 3.1 and 3.2. Also shown are the single XbaI site and the positions of fragments S5 and S7 (see Figure 1.4).

The location of genes identified in this chapter are marked; oriV and Inc from pCRS5; sog from pCRS1a; oriT from pCRS3. The arrow indicates the direction of transcription of a gene. For reference, the site of the eex, ssb and cib genes are also included.

2.35 kb PstI fragment in all of these subcloned plasmids implies that some part of the essential replication region of ColIb either spans the PstI site at coordinate 7.47 or by analogy with some other low copy number origins, a positively required transacting factor may be located on this fragment adjacent to the origin.

3.4 Summary.

The data presented in this chapter can now be summarised in a restriction map of ColIb (Figure 3.4). Although this is presented as a complete map it does not preclude the existence of other small (0.3 kb or less) restriction fragments which have not yet been identified. One way to identify all of these is to perform a Smith-Bernstiel (1976) mapping procedure on the subcloned fragments of ColIb. However the map obtained to date is perfectly adequate for the purposes of this study. Superimposed on this map is the location of those genes or regions which have been identified. The complete map has been used as a basis for locating the site of insertion of Tn5 and Tn1723 described in the next chapter, and for determining the region of ColIb DNA contained in cosmid recombinants (Chapter 7). The knowledge of the location of the genes so far described has aided in the analysis of the phenotype of both of these types of constructions.

CHAPTER FOUR : Construction of Tn1723 and Tn5 insertion mutants of ColIb-P9drd-1

Introduction

Transposon insertion analysis has been used to help elucidate the genetic organisation of many conjugation systems (RP4: Barth et al., 1978; pKM101: Winans and Walker, 1985b; pCU1: Thatte et al., 1985; F: Achtman et al., 1982). This method has three advantages over other ways of constructing point mutations in a conjugation system. Firstly, the introduction of an antibiotic resistance into the plasmid gives a quick phenotypic screen for the insertion event. Secondly, the physical position of the insertion can be easily deduced using restriction analysis. Thirdly, most transposon insertions not only inactivate the gene in to which they are inserted, but are often polar on downstream genes in the same transcriptional unit (Schmitt et al., 1985; Berg et al., 1980, but see Manning et al., 1982). This can give some indication of the organisation of the transfer genes in to operons.

The transposons chosen both carried a kanamycin-resistance determinant specifying neomycin phosphotransferase (see Foster, 1983). Tn1723 was chosen by virtue of the fact that it has an EcoRI site 15 bp from each end of the 9.6 kb element (Ubben and Schmitt, 1985). Now that the EcoRI map of ColIb was complete, it was possible to precisely locate the position of these insertions. The second transposon, Tn5, lacks any EcoRI sites. Therefore, by linearising the plasmid DNA with EcoRI, the transposon can be introduced in to a ColIbdrd-1 parent plasmid at specific sites by the homologous recombination method of Winans and Walker (1983). The isolation of these inserts and their phenotypes are described in detail in the following sections.

4.1 Isolation of the Tn1723 insertion mutants

Initially, ColIbdrd-1 was introduced into the strain RU2537 by conjugation, identifying transconjugants by their ability to produce colicin Ib (RU2537 carries Tn1723 in its chromosome). To isolate transposon-containing derivatives of this plasmid, RU2537(ColIbdrd-1) was used as a donor in a 90 minute liquid mating, using either BW85 or BW86 as the recipient strain. Selection with kanamycin (for Tn1723) and streptomycin (against RU2537) identified recipient cells which had received a copy of ColIb that carried the transposon in the plasmid. These strains were screened for mutations in various phenotypes associated with conjugation, and all plasmids carrying Tn1723 were given the plasmid name pCR(X).

Isolation of transposon insertions in this way, relies on successful conjugal transfer to isolate the Tra$^-$ mutants. This does not preclude the isolation of insertions in the transfer genes however, as illustrated by Barth et al., (1978) who constructed tra::Tn7 insertions of the plasmid RP4 by this method. It is thought that either transposition occurs after conjugation has been triggered or that intact copies of the plasmid present in the donor cell can complement any affected tra genes in trans.

Selection of transfer-deficient transconjugants.

Several matings between RU2537(ColIbdrd-1) and BW85 were performed in parallel and only one transfer deficient (Tra$^-$) transconjugant taken from each one for further analysis. (This was done to try and avoid the possibility of isolating sibling strains.) Transfer deficiency was detected by patch plating transconjugants on to non-selective agar and allowing colonies to develop over night. Next day they were replica plated on to plates containing Nal (50 $\mu g.ml^{-1}$) and Km (50 $\mu g.ml^{-1}$),

TABLE 4.1.1 : EFFICIENCY OF TRANSFER OF Tn1723 INSERTION MUTANTS OF ColIbdrd-1.

Strain	Transconjugants/ml	Relative Transfer Efficiency
BW85(pCR1)	4.0×10^{1}	1.5×10^{-7}
BW85(pCR2)	1.6×10^{8}	0.59
BW85(pCR3)	4.5×10^{3}	1.7×10^{-5}
BW85(pCR4)	4.0×10^{4}	1.5×10^{-4}
BW85(pCR5)	1.0×10^{3}	3.7×10^{-6}
BW85(pCR6)	4.3×10^{3}	1.6×10^{-5}
BW85(pCR7)	9.1×10^{3}	3.4×10^{-5}
BW85(pCR8)	2.1×10^{8}	0.77
BW85(pCR9)	2.7×10^{8}	1.0
BW85(pLG221)	1.8×10^{8}	-

The transfer efficiency in each case was determined in a 1 hour liquid mating using BW97 as the recipient strain and selecting transconjugants on L-agar plates containing 50 $\mu g.ml^{-1}$ Km and 50 $\mu g.ml^{-1}$ Nal. The efficiency is given relative to the number of transconjugants per ml, per hour, achieved by the donor strain BW85(pCR9). The plasmid pLG221 is ColIbdrd-1 ::Tn5 that is standardly used as a Tra^{+} control.

which had been spread with 0.1 ml of an exponentially growing culture of BW97 immediately prior to replica plating, and these plates were incubated over night. Where rapid transfer of plasmid DNA in to the Nal^R recipient had occurred, a patch of transconjugant cells appeared. Transfer-deficient donor strains were unable to form transconjugants sufficiently rapidly and no transconjugant colonies developed. The efficiency of transfer of eight putative Tra^- plasmids from the initial screening was determined more accurately in a 1 hour liquid mating using BW97 as the recipient strain (Table 4.1.1). Of these, six were found to be transfer deficient to varying degrees. None was completely transfer defective (Tra^o); the most defective was pCR1. Plasmids pCR2 and pCR8 were dis arded at this point as they proved to be phenotypically Tra^+. The plasmid pCR9 was taken to be used as a ColIb*drd-1*::Tn*1723* *tra*$^+$ control. Identification of the effects of these insertion on other transfer associated phenotypes are described in Chapter 5 and mapping of the site of the insertion in each case is described in Section 4.2.

Selection of primase-deficient transconjugants.

The expression of the *sog* gene is coordinately regulated with the transfer genes. Therefore it was thought that the selection of Sog^- transposon mutants, which are readily characterized, would provide an alternative method for identifying transfer-defective mutants of ColIb.

The same mating procedure used to isolate the Tra^- mutants was used to isolate Sog^- plasmids, except that the recipient strain was BW86 and the mating was performed at the permissive temperature for this strain (30^oC). Kanamycin-resistant transconjugants were screened for their ability to suppress the temperature-sensitive *dnaG3* mutation of the BW86 host strain by streaking them to single colonies on nutrient agar plates prewarmed to 40^oC. Six strains were chosen which seemed to be primase deficient. These were tested more quantitatively using the test for

TABLE 4.1.2 : COLONY-FORMING ABILITY OF BW86 CARRYING Tn1723 INSERTION MUTANTS OF ColIbdrd-1.

Strain	Colony-forming units per ml at 30°C	Colony-forming units per ml at 40°C	Colony-forming ability
BW86	1.5×10^{8}	0	$<6.7 \times 10^{-9}$
BW86(pCR9)	5.1×10^{7}	2.6×10^{7}	0.51
BW86(pCR10)	1.3×10^{8}	1.0×10^{1}	7.7×10^{-8}
BW86(pCR11)	8.1×10^{7}	1.0×10^{1}	1.2×10^{-7}
BW86(pCR12)	1.1×10^{8}	1.0×10^{4}	9.1×10^{-5}
BW86(pCR13)	9.0×10^{7}	7.5×10^{3}	8.3×10^{-5}
BW86(pCR14)	3.9×10^{7}	2.1×10^{2}	5.4×10^{-6}
BW86(pCR15)	1.0×10^{8}	1.8×10^{4}	1.8×10^{-4}

The colony-forming ability was determined by plating cells on L-agar plates prewarmed to 30°C and 40°C. The colony-forming ability represents the relative survival of cells at the non-permissive temperature for the temperature-sensitive *dnaG3* mutation of the host strain, and is an indication of *sog* primase expression.

colony-forming ability at 40°C (Section 2.3.4). Varying degrees of reduced primase activity were seen (Table 4.1.2) and the plasmids could be divided in to two groups on the basis of this. Plasmids pCR15, pCR12 and pCR13 gave a higher level of supression than plasmids pCR14, pCR10 and pCR11, and which seemed to form a distinct group.. None of the values given were as low as the host strain alone and the figure given by pCR9 approached that given for ColIb*drd-1* (Wilkins, 1975).

Before further analysis of either of the two groups of plasmids isolated could be undertaken, it was important to determine the exact position of the transposons so that the physical location could be correlated with their mutant phenotypes.

4.2 Mapping of the Tn1723 insertions in ColIbdrd-1.

Three restriction enzymes could be used to locate the position of the Tn*1723* insertion sites. These were the enzymes *SalI*, *HindIII* and *EcoRI* for which restriction maps of ColIb had been published by Uemura and Mizobuchi (1982). Sites for all three of these enzymes also exist in Tn*1723* (Figure 4.2.1). Single digests of all the pCR plasmids were performed and the relevant restriction data are displayed in Tables 4.2.1 - 3, see also Figure 4.2.2. Analysis of these data allowed the insertion sites to be mapped to within 0.1 - 0.5 kb, depending on the size of the new restriction fragments generated from the sites within the transposon. The *EcoRI* data gave two possible positions for the insertions, with the smallest fragment generated representing the distance from the end of the transposon to one end of the affected ColIb fragment. The *SalI* and *HindIII* data also allowed two possible positions in each fragment, because the transposon could be in one of two orientations. When all these possibilities were compared they were found to coincide at only one point and this was taken as the point of insertion. Detailed analysis of

FIGURE 4.2.1 : Map of Tn1723 and its location in pCR1, pCR7 and pCR12.

The restriction map of Tn1723 is based on data from R. Schmitt (personal communication). Coordinates below the map are in kb. The location and direction of transcription of the tnpR (resolvase) and tnpA (transposase) is indicated by the arrows above the map.

The distances between restriction sites corresponds to the data given in Tables 4.2.1 and 4.2.2 for these plasmids. In each case the ColIb restriction sites and fragment numbers surrounding the insertion sites are shown. The orientation of the transposon is indicated by an arrow representing the direction of transcription of tnpR and tnpA.

pCR1 : The EcoRI data showed that the insertion was 5.2 kb from one end of one of the two ~20 kb EcoRI fragments of ColIb. The SalI digest showed that the insertion was in S5, which overlaps with the right end of E2. To produce a 7.9 kb SalI restriction fragment, the transposon must be in the orientation shown.

pCR7 : Again the EcoRI data showed that the insertion was in one of the two ~20 kb EcoRI fragments of ColIb. The SalI fragment affected was S1, therefore the insertion must be 2.65 kb from the left end of E2. The insertion is therefore 9.55 kb from the right end of S1 and requires a further 3.05 kb to produce the observed 14.04 kb SalI restriction fragment. This orientates the transposon as shown.

pCR12 : This insertion was also found to be either in E1 or E2, but in this case the S7 fragment was also affected. To produce the 6.6 kb SalI restriction fragment the transposon had to be orientated with the Km^R determinant to the left, 2.1 kb from the right end of E2.

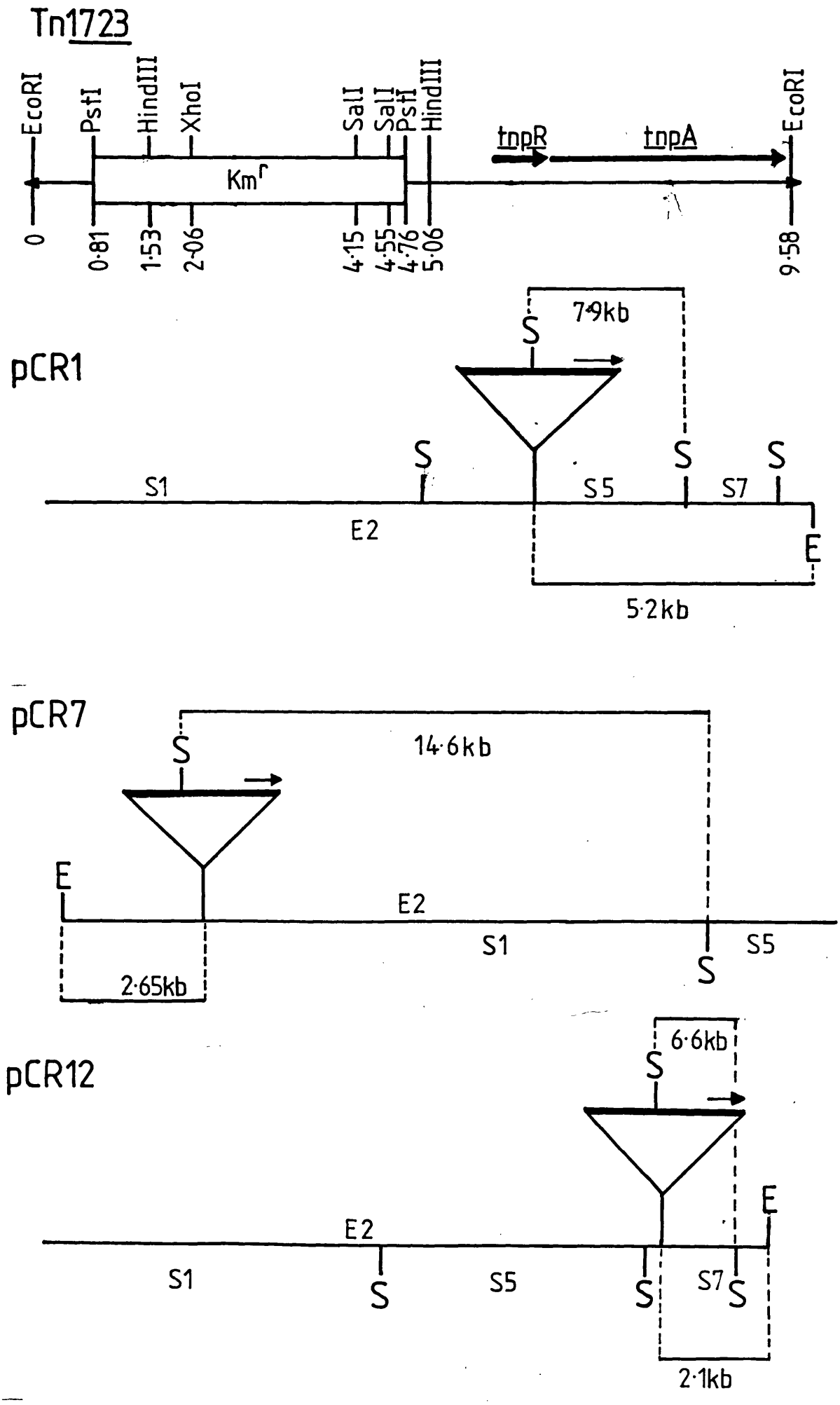

Tn1723
EcoRI
PstI
HindIII
XhoI
SalI
SalI
PstI
HindIII
EcoRI
tnpR
tnpA
Km^r
0
0·81
1·53
2·06
4·15
4·55
4·76
5·06
9·58
pCR1
7·9kb
S
S
S
S
S1
S5
S7
E2
E
5·2kb
pCR7
14·6kb
S
E
E2
S1
S
S5
2·65kb
pCR12
6·6kb
S
E
E2
S1
S
S5
S
S7
S
2·1kb

TABLE 4.2.1 : EcoRI RESTRICTION DATA FOR pCR PLASMIDS.

Plasmid	ColIb fragment cleaved[a]	Size of new restriction fragments (kb)[b]	
pCR1	E2	5.2	~14.5
pCR3	E2	2.69	~16.9
pCR4	E2	2.7	~17.0
pCR5	E14(?)	5.55	3.35
pCR6	E2	2.7	~17.0
pCR7	E2	2.65	~14.5
pCR9	E12	2.5	(0.4)[c]
pCR10	E2	5.8	~13.8
pCR11	E2	4.7	~15.0
pCR12	E2	2.1	~18.0
pCR13	E2	2.1	~18.0
pCR14	E2	6.6	~13.0
pCR15	E2	9.0	~15.0

a: E2 = 19.6 kb, E12 = 2.7 kb, E14 = 2.2 kb.

b: Tn1723 is almost exactly excised by EcoRI, leaving only 15 bp attached to the plasmid DNA and generating a 9.58 kb transposon band

c: Numbers given in brackets indicate sizes of fragments inferred from the physical map.

TABLE 4.2.2 : SalI RESTRICTION DATA FOR pCR PLASMIDS.

Plasmid	ColIb fragment cleaved[a]	Size of new restriction fragments (kb)[b]			
pCR1	S5	7.9	6.45		
pCR3	S1	15.0	~25.0		
pCR4	S1	13.1	~25.0		
pCR5	S3	15.5	12.7	3.75	5.65
PCR6	S1	13.0	~25.0		
pCR7	S1	14.6	~25.0		
pCR9	S2	8.8	~27.0		
pCR10	S5	8.8	5.15		
pCR11	S5	8.05	5.9		
pCR12	S7	6.6	4.5		
pCR13	S7	5.7	5.4		
pCR14	S5	7.91	6.1		
pCR15	S1	5.3	~35.0		

a: S1 = 37.1 kb, S2 = 23.0 kb, S5 = 5.0 kb, S7 = 1.85 kb.

b: Tn1723 contains SalI restriction fragments of 5.03 kb, 4.15 kb and an internal fragment of 0.4 kb.

TABLE 4.2.3 : HindIII RESTRICTION DATA FOR pCR PLASMIDS.

Plasmid	ColIb fragment[a] cleaved	Size of new resticted fragments (kb)[b]		
pCR1	H1	16.5	~30	
pCR3	H1	23.5(D)		
pCR4	H1	22.0(D)		
pCR5[c]	H2	23.0	12.3	5.75
pCR6	H1	22.0(D)		
pCR7	H1	23.0		
pCR9	H1	7.6	~35	
pCR10	H1	17.0	~30	
pCR11	H1	12.3	~30	
pCR12	H1	12.8	~30	
pCR13	H1	12.8	~30	
pCR14	H1	14.5	~30	
pCR15	H1	16.5	~30	

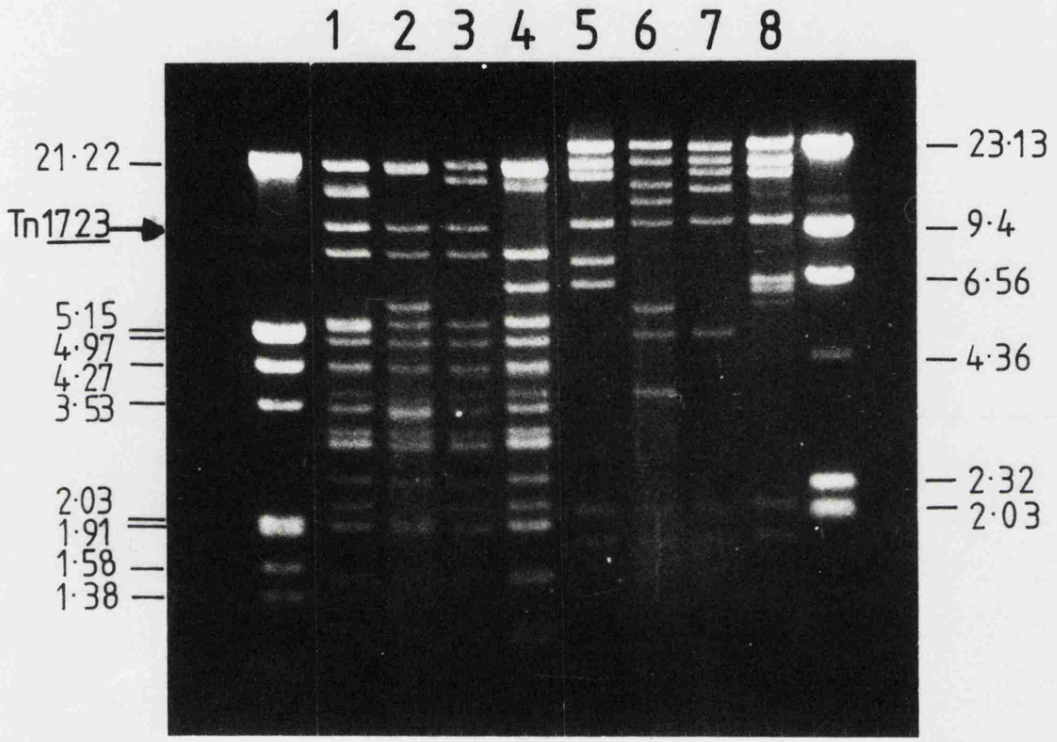
a: H1 = 44.2 kb, H2 = 28.7 kb.

b: Tn1723 has HindIII restriction fragments of 4.52 kb, 1.53 kb and an internal band of 3.52 kb.

c: Note that the HindIII digest of pCR5 gives three new bands.

(D) = doublet band.

FIGURE 4.2.2 : RESTRICTION DIGESTS OF pCR PLASMIDS



Restriction fragments were separated on a 0.75% agarose gel, run at 30 V for 16 hours. Lanes 1 - 4 contain respectively pCR1, pCR5, pCR7 and ColIbdrd-1 DNA cleaved with EcoR1 . Lanes 5 - 8 contain respectively pCR1, pCR5, pCR7 and ColIbdrd-1 DNA cleaved with SalI. The 9.52 kb band corresponding to the Tn1723 element in the EcoRI digests of pCR1, pCR5 and pCR7 is indicated with an arrow.

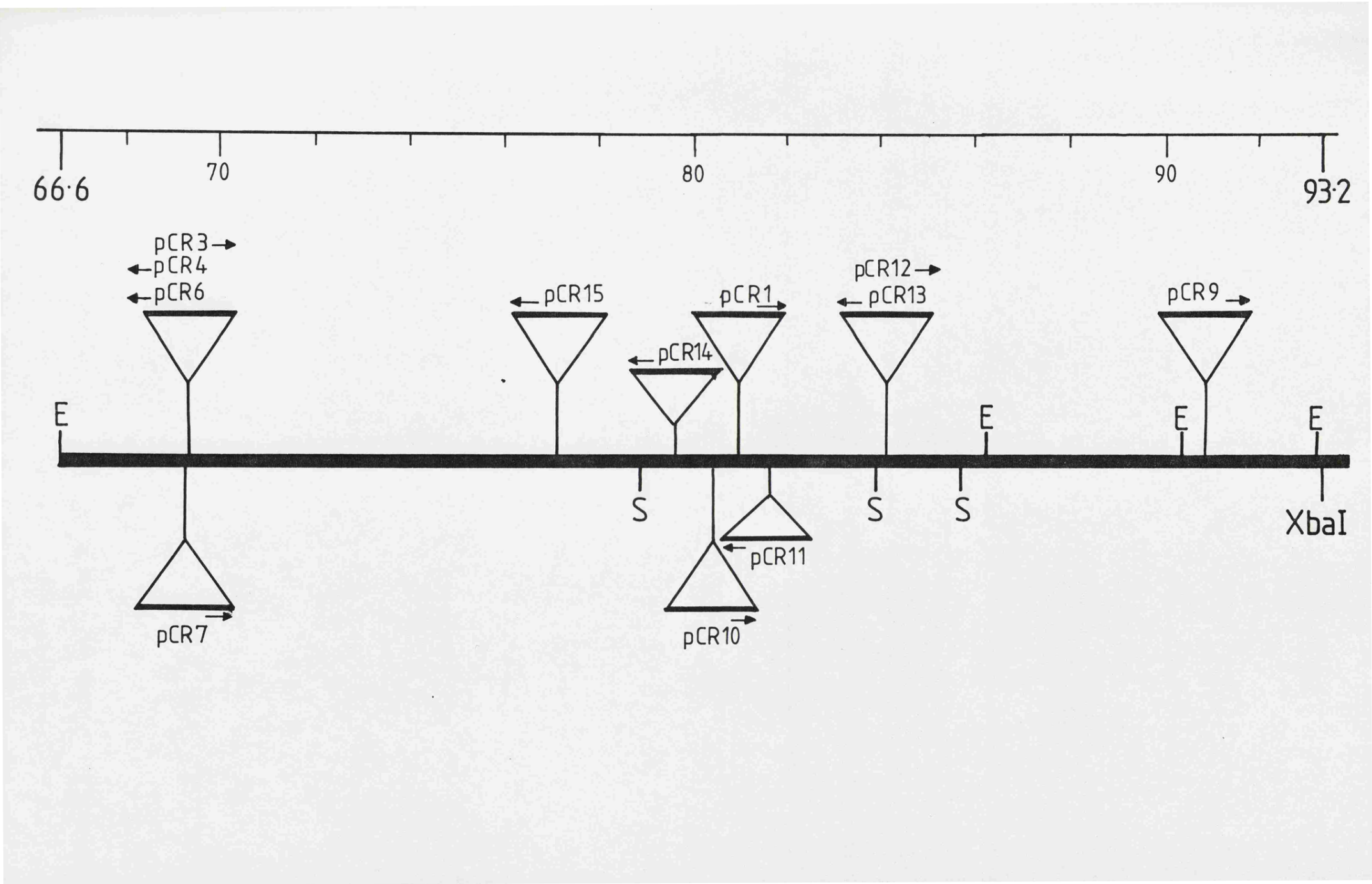
three such insertions are shown in Figure 4.2.1 (pCR1, pCR7 and pCR12). The positions of these and of the remaining Tn1723 insertions are summarised in Figure 4.2.3.

The locations of the transposons in plasmids pCR10 to pCR15 were somewhat suprising in that they were isolated as sog defective plasmids and yet none was found near the known position of this gene in E3. It was possible however that these insertions were exerting polar affects on the sog gene, and that the tra genes were organised in to a long transfer operon. However, despite the direction of transcription of the sog gene from ColIb being in accordance with this model, evidence that the promoter for the expression of sog is included on pCRS1a suggests that this model is not valid. The nature of the affect of these insertions on the sog gene expression is discussed in more detail in Chapter 5.

Only the position of the insertion in plasmid pCR5 could not be mapped. From the EcoRI restriction pattern it would seem that the insertion event has occurred in E14 because this band was absent. However the only new bands visible are both larger than the 2.2 kb of E14 and so cannot be the result of a simple insertion of Tn1723 in to this fragment. Again with SalI and HindIII, more bands were generated than was expected. One possible explanation is that a double insertion event has occurred, but the band which corresponds to DNA solely from Tn1723 is of single intensity only, indicating that just one intact transposon is present. If a double insertion has occurred, it must be a complex event whereby one transposon is inserted in to another. Even to accommodate such a model, a concomitant deletion must have occurred to produce restriction fragments of the sizes seen. The data clearly indicates that the transposon is located in S3, and therefore near oriT. This is predicted to be a region that contains some conjugation genes and the fact that pCR5 is found to be transfer defective lends support to this argument. However from the data available it is not possible to

FIGURE 4.2.3 : Summary map of the insertion sites of Tn1723 in ColIbdrd-1.

The figures given above the map correspond to the kilobase coordinates of ColIb. The restriction sites shown are for EcoRI (E), SalI (S) and the single XbaI site. The orientation of the transposon in each case is indicated by an arrow which represents the direction of transcription of tnpR and tnpA (see Figure 4.2.1). The plasmid names above each insertion site corresponds to the plasmid which carries Tn1723 at that site.


66·6
70
80
90
93·2
pCR3
pCR4
pCR6
pCR15
pCR14
pCR1
pCR12
pCR13
pCR9
E
E
E
E
S
S
S
XbaI
pCR11
pCR7
pCR10

precisely locate this transposon and therefore the position of these genes remains obscure.

The insertion in pCR9, the Tra^+ control, was mapped to E12, the fragment which has also been shown to contain the colicin Ib gene (*cib*) (Boulnois, 1981). The strain carrying this plasmid was tested for colicin Ib production by the method described in Chapter 2 and was found to be *cib*$^+$. The site of this insertion is also shown in Figure 4.2.3

From the positions of these thirteen independently isolated transposon insertions we can see that clustering has occurred in two regions, one in S7 and the other near the end of E2. This may illustrate the so called hotspotting of transposons, where some sites of insertion seem to be preferred. It has been reported that transposons of the Tn*1721* series insert preferentially into A + T rich regions (Ubben and Schmitt, 1985). This may explain the clustering of insertions seen in E2 and although some of these insertions have been mapped to identical sites, the data is not accurate enough to distinguish whether the exact site of insertion is the same in each case or if they encompass a small region of DNA. Whether it is the presence of some consensus sequence for insertion or some other structural feature of the DNA that attracts the transposon remains unclear.

An alternative explanation for this distribution of transposon insertions is that mutations in this region of the plasmid are selected for by the method used to isolate them. Either way, it is possible to overcome this problem by limiting the amount of DNA available in to which transposition can occur. Such a method was used to generate several Tn*5* insertions in specific regions that were predicted to contain conjugation genes.

4.3 Generating Tn5-site specific insertions in ColIbdrd-1.

The principle of this method was originally described by Winans and Walker (1983) and initially involves isolating Tn*5* insertions in a cloned fragment of the region under study. The insertion mutations can then be introduced in to the parental plasmid by homologous recombination in a *recBC sbcB* strain. The large fragment of ColIb cloned in pCRS3 was ideal for introducing Tn*5* in to the region surrounding *oriT*, but other cloned fragments were needed from other parts of the plasmid, especially from the region between the insertion site in pCR7 and the *eex* determinant (coordinates 42.0 to 70.0). For this purpose a bank of *EcoRI* clones of ColIb*drd-1* was generated using the vector pBR328. Plasmids were identified containing inserted fragments of 5.0 kb, 3.6 kb and 3.1 kb which corresponded to fragments E5, E8, and E10 of ColIb respectively. Tn*5* was intoduced in to these fragments by infecting bacteria carrying the recombinant plasmids with the phage λb_{221} *rex*::Tn*5* *Oam*. The amber mutation in the O gene prevents the phage from replicating unless it is in an appropriate translational suppressor strain. The phage cannot integrate in to the host chromosome because the b_{221} deletion removes the *att* site. Therefore after infection the only way that the kanamycin resistance determinant can be maintained is by recombination or transposition in to either the plasmid or in to the host cell chromosome. To distinguish kanamycin-resistant transfectants that contained the transposon in the plasmid from those where it was in the chromosome, plasmid DNA was prepared and used to transform BW85 selecting for acquisiton of both of the plasmid markers and the kanamycin resistance determinant (selection for vector markers reduced the number of plasmids isolated that had Tn*5* inserted in to the vector DNA). The positions of the insertions were then determined by restriction analysis.

FIGURE 4.3.1

A : Restriction map of Tn5

Relevant restiction sites are shown (data taken from Auerswald et al. (1980) and Beck et al. (1982). Also marked are the IS50 insertion sequences that flank the Tn5 element.

B : Determination of the position of Tn5 in pCRS3::Tn5.1.

EcoRI digest : Gain of a 9.55 kb band with concomitant loss of the 3.85 kb band indicated that Tn5 was inserted into the 3.85 kb EcoRI fragment which contains DNA from E9 and pBR328.

EcoRI + HindIII digest : The 2.64 kb band was cut to produce two bands of 2.45 kb and 2.29 kb. The HindIII sites are 1.05 kb from the end of Tn5, therefore the insertion is at position (a) or (b).

SalI digest : The S3 DNA is cut to produce bands of 17.9 kb and 3.77 kb. Therefore the distance from the end of S3 to the transposon is either 0.72 kb (3.77 - 3.05) or 1.26 kb (3.77 - 2.51). The distance from site (a) to the end of S3 is 0.75 kb and the distance from site (b) to the end of S3 is 0.9 kb. Therefore the transposon is inserted at site (a) in the orientation shown (R and L correspond to IS50R and IS50L respectively).

A similar logic was used to map the site of all the Tn5 insertions shown in Figure 4.3.2.

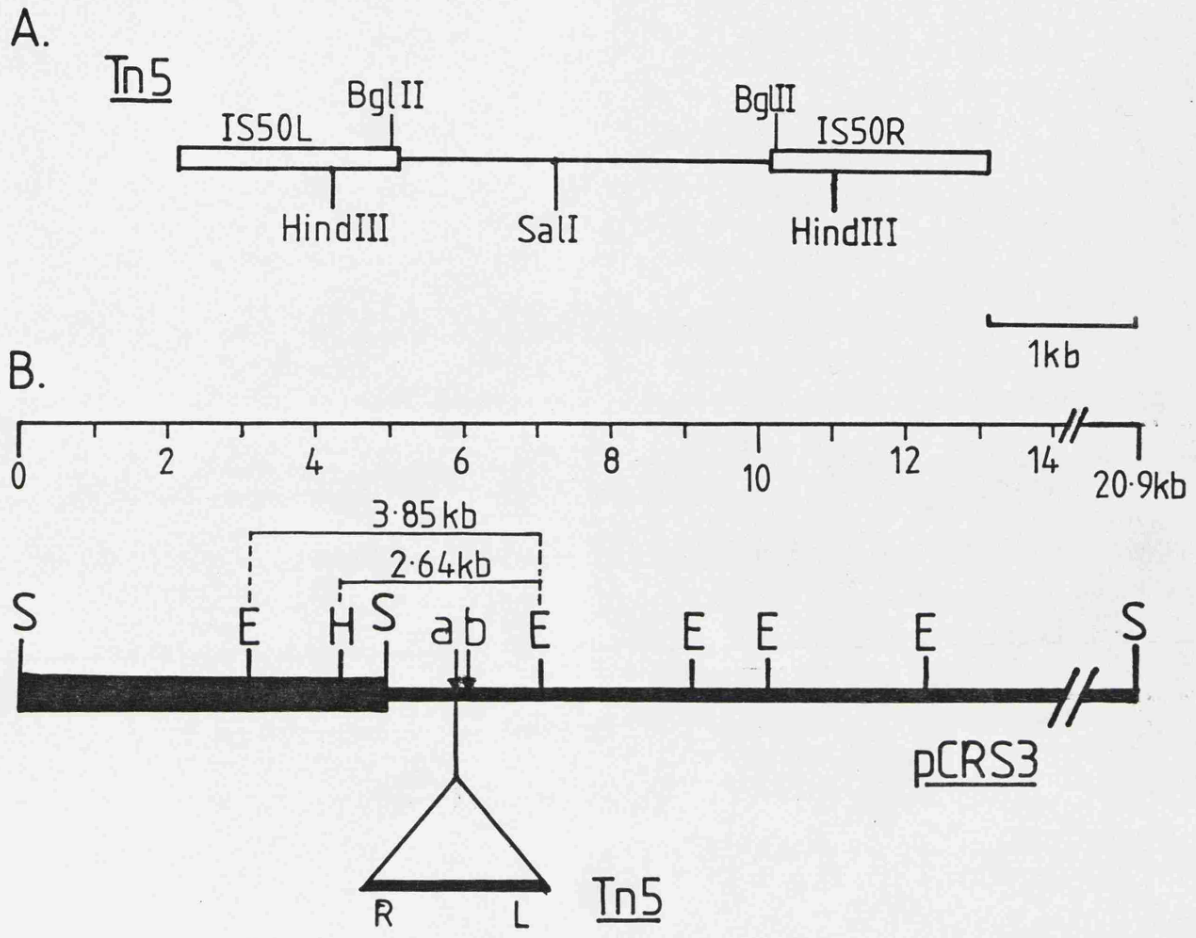

A.
Tn5
IS50L
BglII
HindIII
SalI
BglII
IS50R
HindIII
B.
1kb
0
2
4
6
8
10
12
14
20·9kb
3·85kb
2·64kb
S
E
H
S
a
b
E
E
E
E
S
pCRS3
R
L
Tn5

TABLE 4.3.1 : EcoRI RESTRICTION DATA FOR Tn5 INSERTIONS MUTANTS OF pCRS3.

ColIb Mutant[a]	Plasmid	Restriction Fragments (kb)				
-	pCRS3	11.55	3.85	2.24	2.05	1.05
pCIE9	pCRS3::Tn5.1	11.55	9.55[b]	2.24	2.05	1.05
pCIE15	pCRS3::Tn5.2	11.55	3.85	2.24	7.75	1.05
pCIE20	pCRS3::Tn5.3	11.55	3.85	2.24	2.05	6.75
pCIE14	pCRS3::Tn5.4	11.55	3.85	7.94	2.05	1.05
pCIE1a	pCRS3::Tn5.5	17.3	3.85	2.24	2.05	1.05
pCIE1b	pCRS3::Tn5.6	17.3	3.85	2.24	2.05	1.05
pCIE1c	pCRS3::Tn5.7	17.3	3.85	2.24	2.05	1.05

a: Name of the ColIb::Tn5 mutants derived from the insertion in pCRS3.

b: Tn5 contains no EcoRI sites and therefore the insertion event increases the size of one of the pCRS3 bands by 5.7 kb (underlined).

TABLE 4.3.2 : SalI RESTRICTION DATA FOR Tn5 INSERTIONS MUTANTS OF pCRS3.

ColIb Mutant[a]	Plasmid	Restriction Fragments (kb)[b]			
-	pCRS3	16.0		4.95	
pCIE9	pCRS3::Tn5.1	17.9		4.95	3.77
pCIE15	pCRS3::Tn5.2	16.4	5.3	4.95	
pCIE20	pCRS3::Tn5.3	14.5	7.55	4.95	
pCIE14	pCRS3::Tn5.4	12.8	8.95	4.98	
pCIE1a	pCRS3::Tn5.5	13.55	8.16	4.95	
pCIE1b	pCRS3::Tn5.6	15.95	5.75	4.95	
pCIE1c	pCRS3::Tn5.7	17.0		4.95	4.7

a: Name of the ColIb::Tn5 mutants derived from the insertion in pCRS3.

b: Tn5 has a single SalI site generating fragments of 2.51 kb and 3.0 kb.

c: 4.95 kb band is derived solely from the vector DNA.

Agarose gel of restriction digests of pCRS3 insertion mutants.

Lane	DNA	Enzymes
1	pCRS3::Tn5.2	EcoRI + HindIII
2	pCRS3::Tn5.5	EcoRI + HindIII
3	pCRS3	EcoRI + HindIII
4	pCRS3::Tn5.2	EcoRI
5	pCRS3::Tn5.3	EcoRI

The EcoRI + HindIII restriction fragments of pCRS3 are indicated to the left of the gel, showing which fragments correspond to EcoRI fragments of ColIb.

TABLE 4.3.3 : EcoRI + HindIII RESTRICTION DATA FOR Tn5 INSERTION MUTANTS OF pCRS3

Plasmid	Restriction fragments[a] (kb)							
pCRS3	11.55	2.65	2.24	2.05	1.25	1.05		
pCRS3::Tn5.1	11.55	**3.6**	2.45	2.29	2.24	2.0	1.25	1.05
pCRS3::Tn5.2	11.55	**3.6**	2.65	2.24	2.39	1.75	1.25	1.05
pCRS3::Tn5.3	11.55	**3.6**	2.65	2.24	2.05	1.25	1.82	1.33
pCRS3::Tn5.4	11.55	**3.6**	2.65	2.45	2.05	1.90	1.25	1.05
pCRS3::Tn5.5	9.78	3.87	**3.6**	2.65	2.24	2.05	1.25	1.05
pCRS3::Tn5.6	6.85(D)	**3.6**	2.65	2.24	2.05	1.25	1.05	

a: HindIII digestion of Tn5 generates a 3.6 kb internal band (in bold type) and two 1.05 kb fragments from the ends of the element. The new bands generated are underlined.

(D): Indicates a double band.

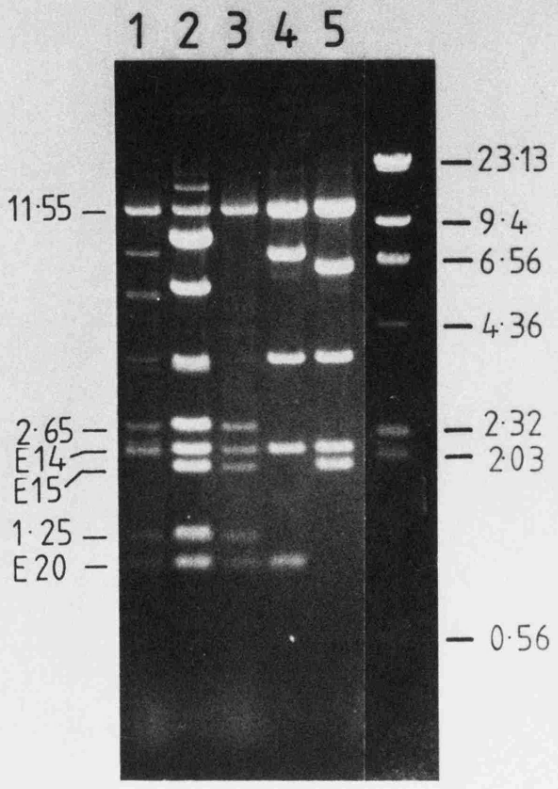
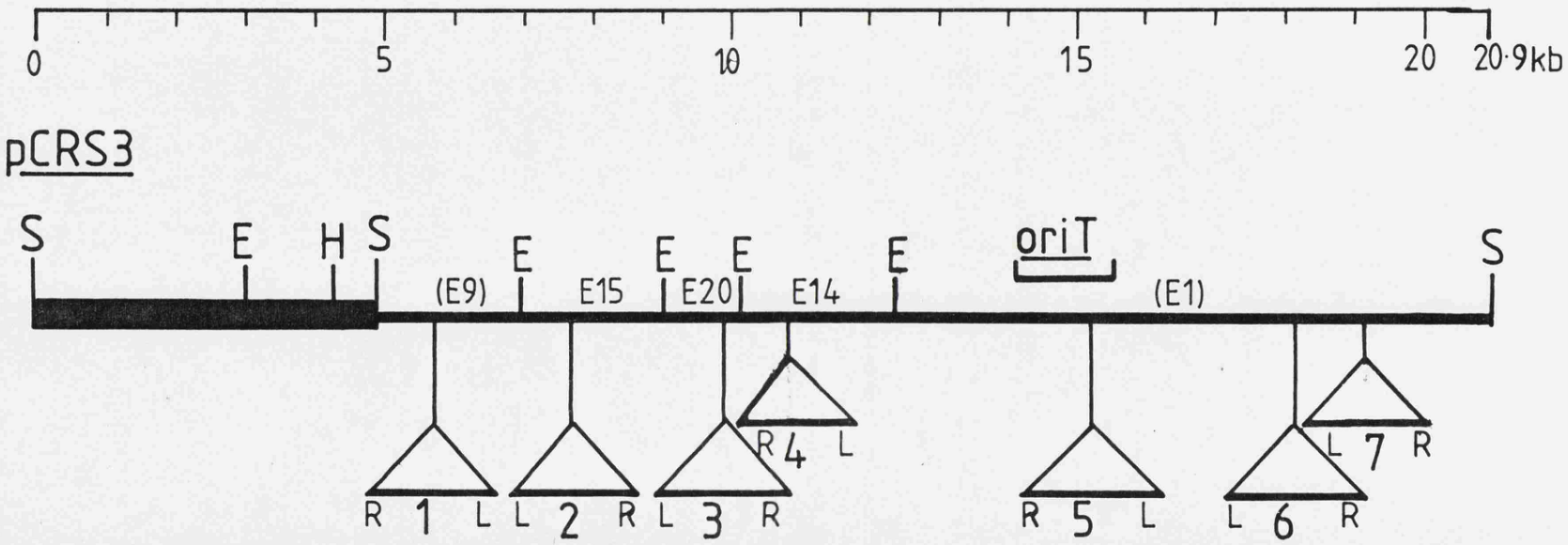


FIGURE 4.3.2 : Position of Tn5 insertions in pCRS3.



The EcoRI fragments of ColIb contained within this fragment are marked, with fragment numbers in brackets representing incomplete EcoRI fragments. The location of the oriT site is also indicated.

The orientation of Tn5 is indicated by the relative position of IS50L (L) and IS50R (R) (see Figure 4.3.1). The number associated with each insertion corresponds to the numbering of pCRS5::Tn5 plasmids in Tables 4.3.1 - 4.3.3.

Position of Tn5 insertions in pCRS3.

The distinctive EcoRI restriction pattern of pCRS3 allowed the site of the Tn5 insertions to be readily identified. The transposon has no EcoRI sites and so the affected band is enlarged by 5.7 kb. The exact position of the insertion within the fragment can be determined using the SalI and HindIII restriction sites in the element (Figure 4.3.1). The data used to position the inserts is given in Tables 4.3.1 - 3 and the location of the transposons is summarised in Figure 4.3.2.

Position of Tn5 insertions in fragments E5, E8 and E10.

Before the position of an insertion in the cloned E5 fragment could be determined, it was necessary to order the HindIII sites in this region of DNA (E5 contains both H4 and H8 of ColIb). The restriction data and a map of the plasmid pCRE5 is presented in Figure 4.3.3. The position of one of the Tn5 insertions in each of the cloned EcoRI fragments was determined as shown in Figure 4.3.4. The logic used to locate these insertions is given in the figure legend and the data is presented in Table 4.3.4.

Introduction of Tn5 in to ColIb*drd-1*.

Once the position of the transposons in the cloned DNA was known, they were ready to be introduced in to the parental plasmid via homologous recombination. ColIb*drd-1* was introduced into the *recBC sbcB* strain N1205. This strain is defective in both exonuclease V (RecBCD enzyme) and exonuclease I (*sbcB* product). Homologous recombination is thought to proceed via the so called RecF pathway, and the absence of these two exonucleolytic enzymes allows the strain to be efficiently transformed with linear DNA (Wackernagel, 1973; Oishi and Cosloy, 1972; for a summary see Kolodner *et al.*, 1985). The plasmids carrying the Tn5 insertions were linearised with the enzyme EcoRI and used to transform

Restriction map of pCRE5.

Restriction sites given are for EcoRI (E), HindIII (H) and SalI (S). Above the map the HindIII restriction fragments of ColIb contained within E5 are shown.

FIGURE 4.3.3.

RESTRICTION DATA FOR pCRE5.

Enzymes	Restriction Fragments (kb)						Total
EcoRI	4.95	4.9					9.85
HindIII + EcoRI	3.6	3.29	1.2	0.6	0.63	(0.46)	9.78
HindIII	4.25	3.29	1.66	0.63			9.83
HindIII + SalI	3.65	3.29	1.66	0.63	0.60		9.83
SalI	~10						
pBR328 x H + S[a]	3.6	1.25					

a: Sizes of fragments generated from the vector by the enzymes HindIII and SalI.

Figures given in brackets represent fragments inferred from the physical map.

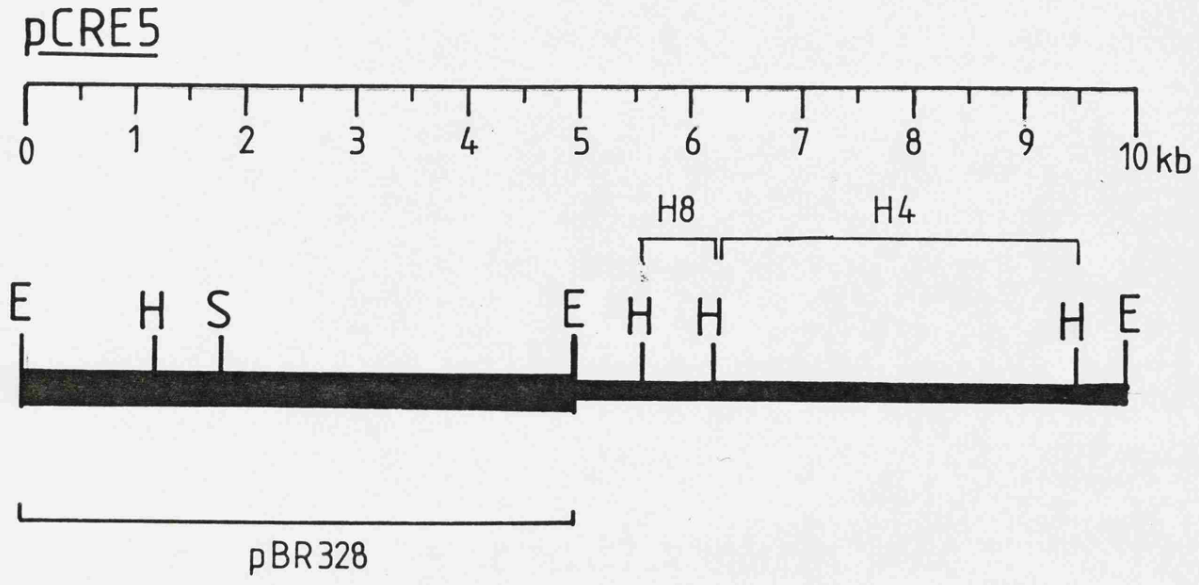
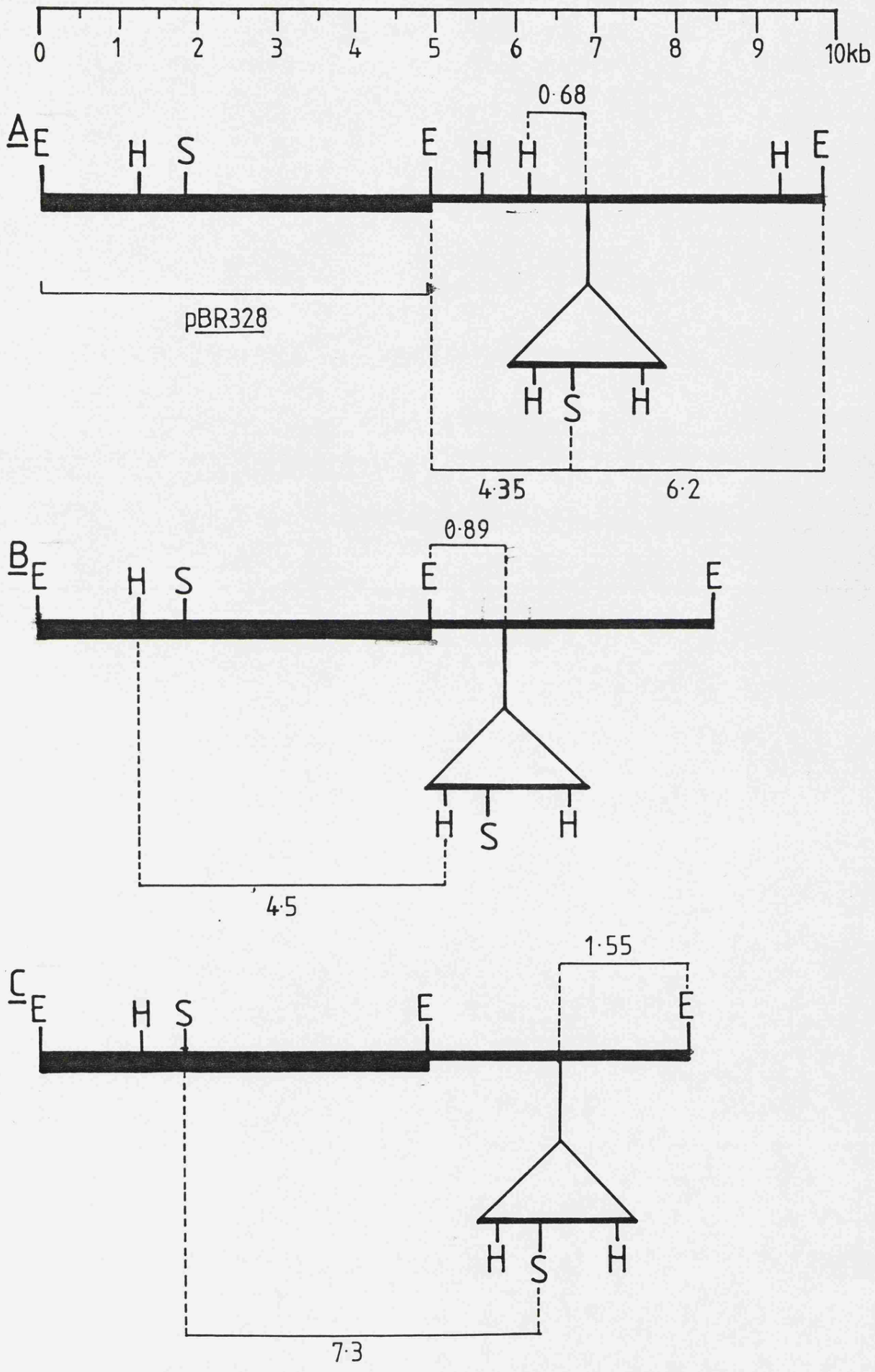


FIGURE 4.3.4 : RESTRICTION MAPPING OF Tn5 INSERTIONS IN EcoRI RESTRICTION FRAGMENT OF ColIb.

A. The HindIII + EcoRI digest showed that Tn5 was inserted in the 3.29 kb HindIII fragment internal to E5, and that it was located 0.68 kb from one end of the 3.29 kb fragment. The EcoRI + SalI digest indicated two possible locations for the insertion, only one of which coincided with the HindIII data at coordinate 6.9.

B. The EcoRI digest indicated that Tn5 was inserted in E8 rather than in the vector DNA. The distance from the HindIII site in the vector to the HindIII site in Tn5 was either 4.5 or 4.1 kb (4.55 - 1.05 or 5.15 - 1.05), and the EcoRI + HindIII digest indicated that the insertion site was only 0.89 kb from one end of the E8 fragment. When compared with the four possible sites allowed from the SalI data, the insertion site was identified at coordinate 5.9, at which point all three sets of data coincided. The orientation of the cloned E8 fragment with respect to the ColIb plasmid is unknown.

C. The HindIII fragments of 6.54 and 3.85 kb were cleaved by EcoRI to give fragments of 2.8 + 3.67 kb and 2.6 + 1.22 kb respectively. The 3.67 and 1.22 kb fragments come from the vector DNA, therefore the transposon can be sited relatively easily. The orientation of the transposon was determined from the SalI restriction data once the distance from the insertion site to the SalI site in the vector was known. Again the orientation of the E10 fragment with respect to the ColIb plasmid was unknown but this insertion was used to generate the plasmid pCRS1a. Mapping of this plasmid showed that the distance from the end of the transposon to the end of the E10 fragment was 1.55 kb and thus the left end of E10 as drawn is next to E8 and the right end, adjacent to E16.


0 1 2 3 4 5 6 7 8 9 10kb
A
E H S E H H H E
0·68
pBR328
H S H
4·35
6·2
B
E H S E E
0·89
H S H
4·5
C
E H S E E
1·55
H S H
7·3

Agarose gel of restriction digests of pCRE10::Tn5.

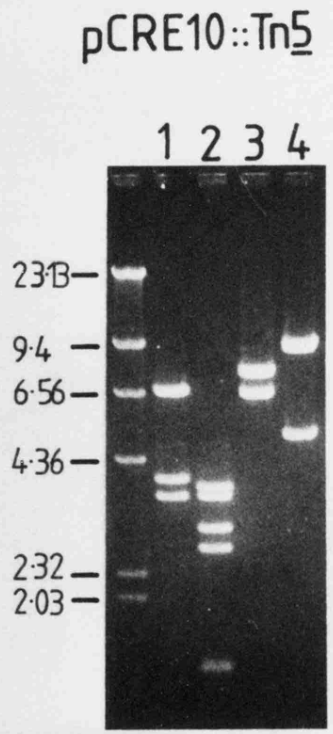
Lane	DNA	Enzymes
1	pCRE10::Tn5	HindIII
2	pCRE10::Tn5	HindIII + EcoRI
3	pCRE10::Tn5	SalI
4	pCRE10::Tn5	EcoRI

0.9% (w/v) agarose gel, run for 20 V for 16 hours.

TABLE 4.3.4 : RESTRICTION DATA FOR Tn5 INSERTION MUTANTS OF pCRE5, pCRE8 AND pCRE10

Plasmid	Enzymes	Restriction Fragments (kb)							Total
pCRE5::Tn5	HindIII + EcoRI	3.65	3.6(D)	1.7	1.2	0.63	0.60	0.46	15.47
	SalI	7.95	7.41						15.36
	SalI + EcoRI	6.2	4.35	3.05	1.85				15.45
	EcoRI	10.5	4.95						15.45
pCRE8::Tn5	HindIII	5.55	5.15	3.6					14.3
	HindIII + EcoRI	5.85	3.75	3.4	1.22				14.22
	SalI	7.9	6.4						14.3
	SalI + EcoRI	6.0	3.4	3.05	1.85				14.3
	EcoRI	9.55	4.95						14.5
pCRE10::Tn5	HindIII	6.45	3.85	3.55					13.85
	HindIII + EcoRI	3.67	3.55	2.8	2.60	1.22			13.84
	SalI	7.3	6.45						13.75
	EcoRI	9.0	4.95						13.95

(D): Indicates a double band.



N1205(ColIbdrd-1), selecting for kanamycin resistant transformants. The linearised plasmid cannot be maintained (even closed circular ColE1-like replicons cannot be maintained in a recBC sbcB double mutant strain: Basset and Kushner, 1984) and therefore these transformants should represent events where Tn5 has been introduced in to ColIb by homologous recombination between DNA on either side of the insertion site.

Plasmid DNA was prepared from the transformants to check that the transposon was in the expected position in the parent plasmid. EcoRI restriction patterns showed a single band to be missing and the concomitant appearance of a new band equivalent in size to the affected fragment plus the 5.7 kb of the transposon (see Figures 4.3.5). Figure 4.3.5A shows the DNA prepared from four independent isolates of a strain transformed with pCRE8 DNA. As can be seen from Lane 1, a transposition event can occur from the transforming DNA (approximately 1 in 8 isolates) such that the transposon now occupies an entirely new site (also reported by Ubben and Schmitt, 1985). One of these random transpositions allowed an insertion in E18 to be isolated (Figure 4.3.5B, track 4). SalI digests of this plasmid indicated that the transposon was in the 0.5 kb of E18 that overlapped with S6 (i.e. to the right of the end of the DNA cloned in pCRS1a, data not shown).

All these plasmids were given pCI numbers corresponding to the fragment in which the transposon was located e.g. pCIE5 = ColIb E5::Tn5 and pCIE20 = ColIb E20::Tn5. (Different insertions in E1 are labelled E1a, E1b and E1c.) Now that a fairly widespread series of transposon insertions were available, a study of their effect on various transfer related phenotypes was undertaken. This will be described in Chapters 5 and 6.

FIGURE 4.3.5 : Restriction digests of Tn5 insertion mutants of ColIbdrd-1.

A : Lanes 1 - 4 contain EcoR1 restrictions of DNA prepared from four independent isolates from the transformation of N1205(ColIbdrd-1) with EcoRI-linearised pE8::Tn5 DNA. The new bands corresponding to E8::Tn5 and E8'::Tn5 are marked, as are the usual positions of E8 and E8' (see Lane 1), which are missing in lanes 2, 3 and 4. In lane 1, the transposon has been inserted into an entirely new site in E1 or E2 (see text).

(0.75% (w/v) agarose gel run at 20 V for 16 hours.)

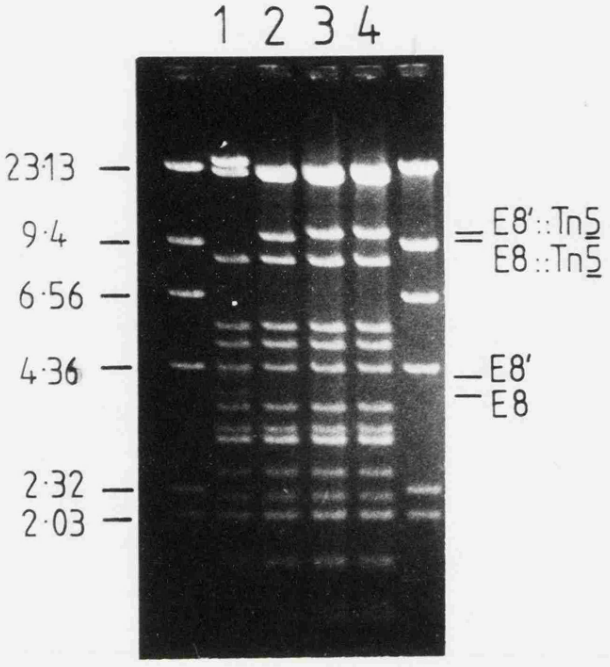
B : EcoRI restriction digests of different ColIbdrd-1 Tn5 insertion mutants:

Lane	Plasmid DNA	EcoRI fragment containing Tn5
1	pCIE8	E8
2	pCIE10	E10
3	pCIE15	E15
4	pCIE18	E18
5	pCIE20	E20

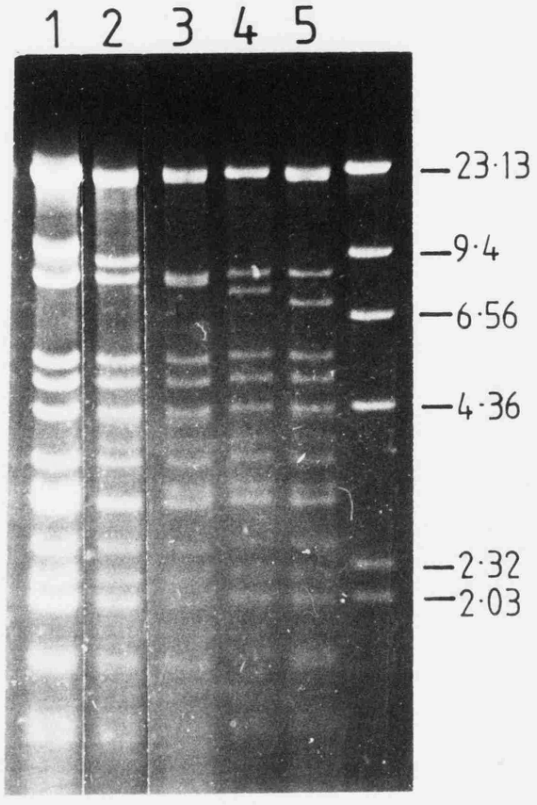
The gain of a new band of 7 - 14 kb can be seen in each case. No concomitant loss of bands is seen for pCIE15, pCIE18 or pCIE20 as their loss is concealed by the presence of the fragments E16, E17 and E19 respectively.

(0.75% agarose gel, run at 25 V for 16 hours.)

A.


1 2 3 4
23·13
9·4
6·56
4·36
2·32
2·03
E8'::Tn5
E8::Tn5
E8'
E8

B.


1 2 3 4 5
23·13
9·4
6·56
4·36
2·32
2·03

CHAPTER FIVE : Characterisation of transposon insertion mutants; identification of a two part regulatory system.

Introduction.

Bradley (1983) showed that plasmids of the IncI complex express both a thin flexible pilus and a thick rigid pilus. Expression of the thin I_1 pilus is easily detected, since it confers sensitivity to the I_1-male specific bacteriophages I_α and PR64FS on the host cell. PR64FS is a filamentous single-stranded DNA phage that adheres to the tips of thin pili (Coetzee et al., 1980), whilst Iα is an RNA phage that adheres to the sides of the pilus (Coetzee et al., 1982). Despite extensive searches by Bradley (1983), no phage has yet been isolated which uses the thick I_1 pilus as a receptor.

At the beginning of this study the only method available to detect the presence of the thick pilus directly was electron microscopy. However this was not a convenient method for screening large numbers of mutant strains. Using antisera raised against R144drd-86 thick pili, prepared by Bradley (1983), an enzyme-linked in situ assay (ELISA) was devised to detect the presence of the thick pilus on cells carrying ColIb plasmids.

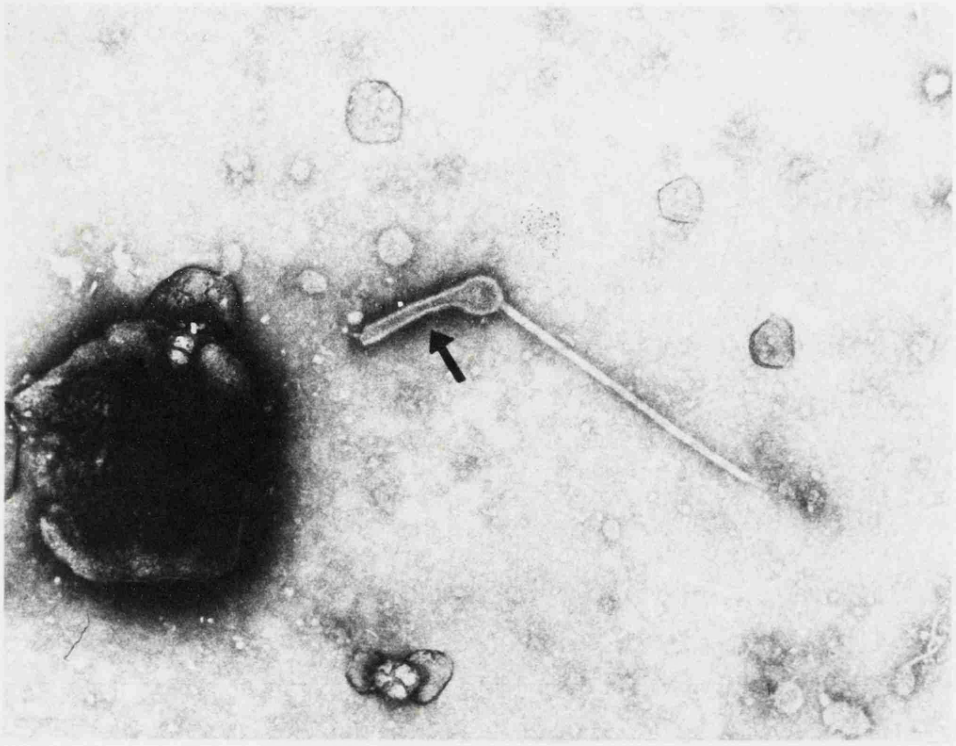
The antibody had been raised against purified pili, but it was also found to cross-react strongly with whole cells of E.coli that did not express thick I_1 pili, probably due to non-specific binding by serum proteins. To try and reduce this background reaction, the antiserum was purified on a Sepharose-protein A column. The staphylococcal protein A binds IgG nonspecifically in neutral conditions (pH 7.5). Once the non-immunoglobulin material had been washed through the column, the antibody was eluted using 0.1 M acetic acid. After this treatment the background reaction with whole cells was much reduced but not totally

FIGURE 5.01 : Electron micrographs of thick I_1 pili prepared from ColIbdrd-1 (from D. Bradley).

A : Thick I_1 pilus, prepared as described in Bradley (1984); 54,000 x magnification. The arrow indicates the basal membrane structure found to be attached to the free pili. The pilus is seen to be a short rigid structure with a pointed distal tip.

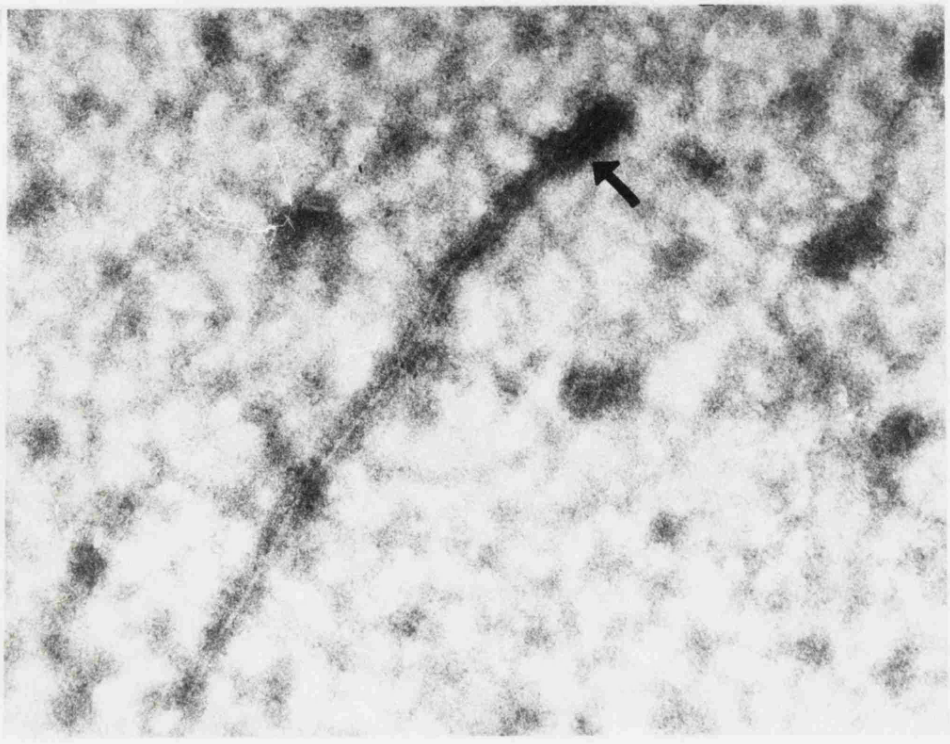
B : Thick pilus treated with anti-R144drd-11 thick pilus antiserum; 53,400 x magnification. Immune electron microscopy was performed as described in Bradley, (1984). Note that the antibody adheres to both the pilus shaft and the basal membrane structure (indicated by the arrow).

A.



x 54,000

B.



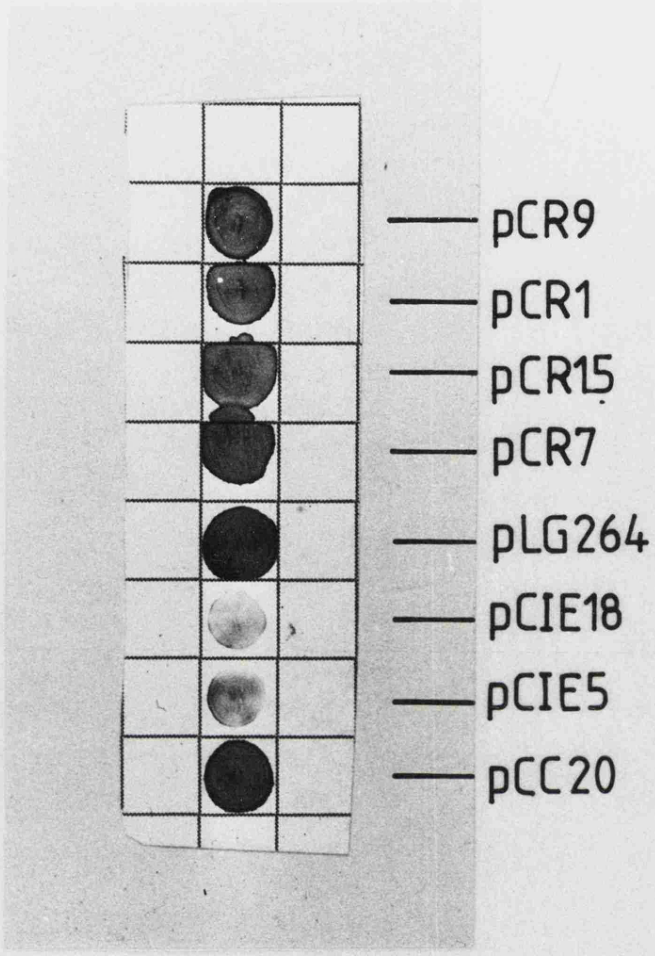
x 53,400

abolished. A second problem existed in that although the antiserum was raised against purified pili, membrane vesicles are often found associated with the basal structures of pili when they have been sheared from whole cells (Bradley, 1984, see Figure 5.01). Therefore this antiserum also contained antibody to E.coli cell membranes. To reduce this problem, cells were grown to exponential phase and then the pili were sheared from the surface of the cell by shaking the culture vigorously. The cells were then removed by centrifugation and the supernatant used in an ELISA type of reaction (see Section 2.3.7 and Figure 5.02). In this way, many different mutants could be quickly screened, but those of interest were sent to Dr D. Bradley for quantitative analysis by electron microscopy.

It has been noted for many conjugation systems that encode short rigid pili that the rate of transfer in liquid matings is lower than when mating occurs on a solid surface (Bradley, 1980b). The cells are thought to be brought in to intimate contact when they are concentrated together on a surface and the absence of shear forces normally present in liquid culture allows the initial pair formation to occur. It is thought that contacts formed by flexible pili are less sensitive to shearing and therefore allow enhanced levels of transfer in liquid culture. The rescue of mating frequency mediated by thin-pilus negative IncI plasmids in a surface mating has been taken to indicate that cells which no longer express the thin flexible I pilus are still elaborating the second type of pilus, the thick rigid type. These two mating procedures combined together provide a way of determining whether a drop in mating efficiency is due solely to the loss of expression of the thin pilus, or if thick pilus synthesis is also affected.

In the following sections the pilus expression and expression of sog primase and mating efficiency of all those mutants so far constructed are described. Also included are data pertaining to other transfer-defective

Figure 5.02 : In situ Enzyme-Linked Immuno Absorption Assay (ELISA).



Test strains were grown to stationary phase and then pili removed from the cell surface by vigorous agitation. Cell debris was removed by centrifugation and 5 µl of supernatant concentrated on to a nitrocellulose strip. The presence of thick I_1 pilin was detected using anti-R144drd-86 thick pilus antiserum (Bradley, 1983), as described in Section 2.3.7. The plasmid in the test strain in each case is indicated on the right. When thick pili were present in the supernatant a dark colour reaction was seen (pCR9 is the Tra^+, $Pilus^+$ control plasmid). The fainter colour reaction given by strains containing pCIE18 and pCIE5, was the same as that given by the plasmid-free host strain and indicated that these plasmids do not determine thick I_1 pili.

plasmids that had been isolated in this laboratory but had only to date been characterized for deficiency in transfer.

5.1 Efficiencies of transfer and pilus expression of Tn1723 and Tn5 insertion mutants of ColIb.

The efficiency of transfer was determined for both liquid and surface matings in a 1 hour mating using a 1 : 1 donor to recipient ratio by the methods described in Chapter 2. Efficiency is measured as the ratio between the number of Km^R transconjugants per ml for the test plasmid and the value given by the Tra^+ control plasmid, pCR9. The results of transfer experiments and the various tests for the expression of the two types of I_1 pilus are summarised in Table 5.1.1.

The plasmids in this table have been grouped together according to the location of the transposon insertion. All the insertions in the region between coordinates 67.0 and 83.0 on the map of ColIb are defective in transfer to varying degrees. Of the insertions in S5, pCR1 and pCR11 were most defective, whilst pCR10 and pCR14 gave slightly higher transfer frequencies. These insertions map within 1 kb of each other, but the limitations of the accuracy of the mapping of insertions that were generated directly in ColIb did not allow these to be considered as insertions at separate sites. The finding that the physical location of the insertions in S5 correlated with the degree of transfer deficiency, suggests that there are at least two separate transfer-related loci in S5, and the decreased severity of the effect of the insertion in pCR10 and pCR14 over those in pCR1 and pCR11 suggests that they may be in one transcriptional unit which reads from right to left. These plasmids were also found not to express the thin I pilus, and both pCR1 and pCR10 expressed reduced levels of thick I pilus, as determined by electron microscopy.

TABLE 5.1.1 : EFFICIENCY OF TRANSFER AND PILUS EXPRESSION OF ColIbdrd-1 DERIVATIVES.

Plasmid	Transconjugants ml^{-1} in liquid	Efficiency	Transconjugants ml^{-1} on solid	Rescue	Thin Pili	Thick Pili
pCR9	2.7×10^{8}	1.0	1.8×10^{8}	0.67	+	drd +
pCR12	1.6×10^{7}	5.9×10^{-2}	ND	ND	+	drd +
pCR13	9.8×10^{6}	3.6×10^{-2}	ND	ND	+	ND +
pCR1	4.0×10^{1}	$1.5 \times 10^{7-}$	1.4×10^{4}	3.5×10^{2}	-	rd +
pCR11	5.0×10^{1}	1.9×10^{-7}	ND	ND	-	ND +
pCR10	2.0×10^{3}	7.4×10^{-6}	ND	ND	-	- +
pCR14	9.3×10^{3}	3.4×10^{-5}	ND	ND	-	ND +
pCR15	6.2×10^{2}	2.3×10^{-6}	5.1×10^{5}	8.3×10^{2}	-	drd +
pCR3	4.5×10^{3}	1.7×10^{-5}	ND	ND	-	ND +
pCR4	4.0×10^{4}	1.5×10^{-4}	ND	ND	-	- +
pCR6	4.3×10^{3}	1.6×10^{-5}	ND	ND	-	ND +
pCR7	9.1×10^{3}	3.4×10^{-5}	2.8×10^{7}	3.0×10^{3}	-	rd +

continued,..

TABLE 5.1.1 continued,..

Plasmid	Transconjugants ml^{-1} in Liquid	Efficiency	Transconjugants ml^{-1} on Solid	Rescue	Thin Pili	Thick Pili	
pCIE8	1.3×10^{8}	0.48	ND	ND	+	ND	+
pCIE10	2.2×10^{8}	0.82	ND	ND	+	ND	+
pCIE5[a]	1.1×10^{2}	4.1×10^{-7}	5.0×10^{1}	--	+	-	-
pCIE18	$<2.7 \times 10^{-8}$	3.7×10^{-9}	$<2.7 \times 10^{-8}$	NIL	-	-	-
pCIE9	1.1×10^{8}	0.41	ND	ND	+	ND	+
pCIE15	1.3×10^{8}	0.48	ND	ND	+	ND	+
pCIE20	1.5×10^{7}	5.6×10^{-2}	ND	ND	+	ND	+
pCIE14	1.2×10^{8}	0.44	ND	ND	+	ND	+
pCIE1a	1.1×10^{8}	0.41	ND	ND	+	ND	+
pCIE1b	1.2×10^{8}	0.44	ND	ND	+	ND	+
pCIE1c	1.5×10^{8}	0.56	ND	ND	+	ND	+
pCR5	1.0×10^{3}	3.7×10^{-6}	ND	ND	+	drd	+

continued,..

Donor and recipient cells were mixed in a 1 : 1 ratio and were incubated together for 1 hour. Transconjugants were selected on L-agar plates containing kanamycin (50 $\mu g.ml^{-1}$) and nalidixic acid (50 $\mu g.ml^{-1}$).

Efficiency is given relative to the frequency of transfer of pCR9 (ColIb*drd-1* E12::Tn*5* Tra^+). Rescue represents the increase in frequency of transfer of each strain in the surface mating system.

Nomeclature used for thick pilus synthesis;

drd : normal *drd* levels of thick pili produced.

rd : few thick pili produced (less than 1 per cell).

\- : no thick pili detectable.

\+ : thick pili produced.

Data in the first of the thick pili columns obtained from electron microscopy (D. Bradley) and in the second from the ELISA test.

The synthesis of thin pili was detected using the phage Iα or PR64FS. Strains that produced thin pili were not tested in the surface mating system.

Only one insertion was isolated in the region containing the transposon in pCR15. This also had a greatly reduced efficiency of transfer but differed significantly from the S5 insertion in that although it also did not produce thin I pili, it scored positively for thick pilus expression both by the ELISA screen and by electron microscopy. What was surprising was the finding that the transfer efficiencies of both pCR1 and pCR15 were rescued equally well by mating on solid media, although the overall level of pCR1 transfer remained lower. This suggests that although the insertions in S5 may cause reduced level of pilus synthesis, sufficient pili are still produced to mediate conjugation.

The group of insertions around that in pCR7 seem to confer identical phenotypic effects despite a slight difference between the location of Tn*1723* in pCR7 to that in pCR3, pCR4 and pCR6 (whether this difference is real or an artifact of mapping is difficult to assess) and the different orientation of the transposons in this region. Once it had been established that the phenotype of these insertion mutants was identical in all cases (see also expression of *sog*, surface exclusion and nuclease in later sections), pCR7 was taken as a representative of this group.

When a strain containing pCR7 was used as a donor in a surface mating system, the frequency of transfer was raised to a value only ten fold lower than the Tra^+ control. Strains carrying pCR7 did not synthesise thin I pili and also had reduced levels of thick I pilus expression. Therefore it is not possible to make a direct correlation between levels of thick I pilus expression and transfer frequency, suggesting that those insertions in S5 affect more functions than pilus synthesis alone. Insertions on both sides of the pCR15 insertion site affect levels of thick I pilus synthesis, whilst this plasmid produces apparently normal thick pili. This suggests that the loci affected by the insertions in S5, pCR15 and pCR7 are probably not contained within one transcriptional

unit, since all three mutations abolish thin pilus expression but have varying effects on thick pilus expression. If the two functions were intimately related (for instance by sharing assembly mechanisms), it would be expected that insertions affecting thin pilus synthesis would also affect thick pilus assembly, however this does not seem to be the case. The effect of these insertions on thick pilus expression may not be directly exerted on the genes required for thick pilus assembly at all. Assuming that pilus assembly occurs at specialized zones of adhesion, a situation may arise where defective thin pilus assembly would block these sites and result in reduced levels of thick pilus synthesis also.

The only other insertions that were defective in pilus synthesis were those in plasmids pCIE5 and pCIE18. Initially strains carrying pCIE5 scored positively in phage tests, producing plaques with both Iα and PR64FS. However the strains rapidly lost this phenotype and at the same time the frequency of transfer mediated by this plasmid dropped from 1.8×10^6 down to 1.1×10^2. When tested for thick pilus synthesis, strains carrying pCIE5 were shown to be pilus negative, both by the ELISA test and by electron microscopy, but the tests were only carried out after the transfer frequency had dropped. This suggested that the plasmid was rapidly accumulating secondary mutations and might be reverting to a drd$^+$ phenotype, possibly because the effect of the insertion was very detrimental to the host bacterium. Attempts to reisolate a drd pCIE5 plasmid by performing matings for only short periods, thereby selecting for the most fertile plasmids, failed and no further studies were performed with this plasmid. The fact that when initially isolated, the strain was already transfer deficient suggests that some tra functions may be affected by this insertion in E5.

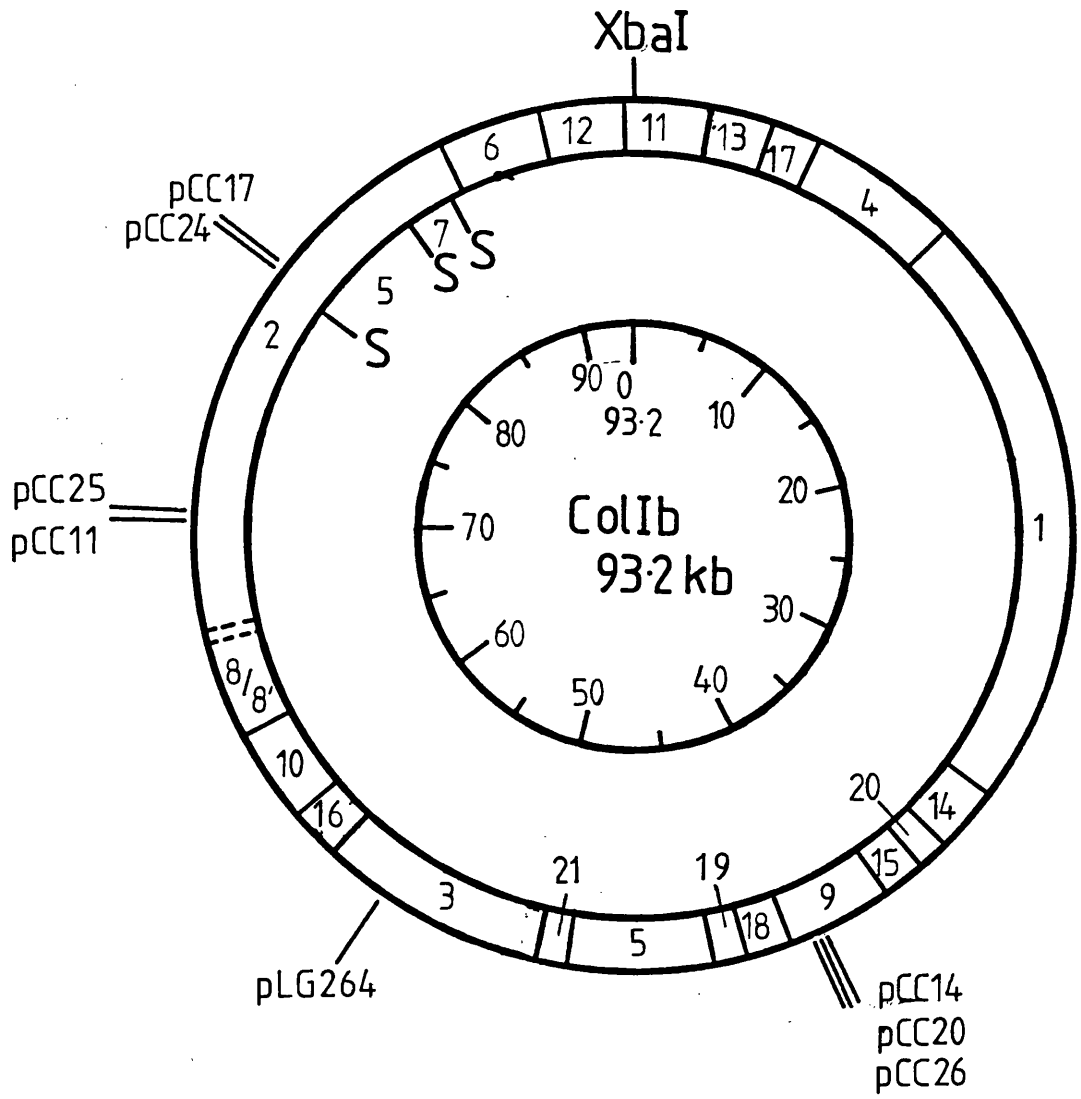
The mutant pCIE18 was the only truly Tra^o plasmid isolated. Even when mating mixtures were concentrated 100 fold on to selective plates, no transconjugants were formed. This plasmid did not express either

thick or thin I pili but unlike pCIE5 this cannot be a *drd*$^+$ revertant, since even repressed ColIb plasmids transfer at a low frequency. The plasmid could be complemented easily *in trans* (see Chapter 7) and so the mutation apparently exerts a direct effect on the synthesis of both types of pilus. Considering that the other regions identified to be involved in thin pilus synthesis were so far from this insertion site, it is possible that the gene affected is required for the assembly of both types of pilus, or it may represent a control factor, such as *traJ* of the F plasmid. A prediction of this is that the insertion would have pleiotropic effects on the expression of all known transfer functions (see sections 5.3 and 5.4 and Chapter 6).

The remaining pCI plasmids all showed normal levels of transfer, and therefore the insertions are not thought to be in essential transfer regions. This leaves a large segment from the exclusion determinant in E9 through to E14 which does not seem to contain any large blocks of transfer genes. Insertions on the other side of *oriT* also did not affect transfer but without saturating the region with transposon insertions, it is impossible to rule out completely the location of some transfer genes in either of these regions. There is evidence from pCR5 (which maps at an unknown point near *oriT*; see Chapter 4) that some transfer function lie between *oriT* and *eex*. One of the insertions in this region did show a small effect on transfer. The plasmid pCIE20 consistently transferred with a 10 fold reduced frequency (the experiment was repeated six times and the same reduced frequency was obtained each time). Such an effect may be caused if the insertion disrupts the expression of a gene which may be complemented by a host cell protein (c.f. the transfer of the primase defective plasmid pLG250 is only 10 fold defective; Chatfield *et al.*, 1982).

In the course of studying other facets of ColIb-mediated conjugation, a second set of transfer-defective Tn*5* insertion mutants of the plasmid

FIGURE 5.1.1 : Summary map of Tn5 insertions in ColIbdrd-2.



Numbers within the circle correspond to EcoRI fragments of ColIb. Also shown is the single XbaI site and the position of fragments S5 and S7. These insertion mutants were mapped by B. Wilkins (unpublished data). The site of each insertion is represented by a single line and the number of the plasmid that has an insertion at that point is indicated in each case.

TABLE 5.1.2 : CHARACTERIZATION OF Tn5 INSERTION MUTANTS OF ColIbdrd-2.

Plasmid	Transconjugants ml^{-1} Liquid	Efficiency	Transconjugants ml^{-1} Solid	Rescue	Thin	Thick	
pCC17	7.2×10^{3}	7.7×10^{-5}	3.9×10^{3}	0.54	-	ND	+
pCC24	9.8×10^{2}	1.0×10^{-5}	2.1×10^{4}	2.1×10^{1}	-	ND	+
pCC11	1.2×10^{5}	1.3×10^{-3}	4.3×10^{7}	3.6×10^{2}	-	drd	+
pCC25	5.9×10^{4}	6.3×10^{-4}	2.1×10^{7}	3.6×10^{2}	-	drd	+
pLG264	1.8×10^{6}	1.9×10^{-2}	4.1×10^{5}	0.23	+	drd	+
pCC14	1.4×10^{2}	1.5×10^{-6}	3.0×10^{2}	2.1	+	+?[a]	+
pCC20	1.3×10^{2}	1.4×10^{-6}	$<10^{1}$	7.7×10^{-2}	+	+?	+
pCC26	6.7×10^{1}	7.1×10^{-7}	$<10^{1}$	0.15	+	+?	+
pLG269	9.4×10^{7}	1.0	5.1×10^{7}	0.54	+	ND	+

a : Number of pili per cell could not be determined; see text.

Nomeclature used for thick pilus synthesis;

drd : normal drd levels of thick pili produced.

rd : few thick pili produced (less than 1 per cell).

- : no thick pili detectable.

+ : thick pili produced.

Data in the first of the thick pili columns obtained from electron microscopy (D. Bradley) and in the second from the ELISA test.

BW86 donor cells and recipients (BW97) were mixed in a ratio of 1 : 9 and were incubated at 31°C for 1 hour. Transconjugants were selected on L-agar plates containing kanamycin (50 $\mu g.ml^{-1}$) and nalidixic acid (50 $\mu g.ml^{-1}$). Efficiency is given relative to the frequency of transfer of pLG269 (ColIbdrd-2::Tn5 Tra^{+}). Rescue represents the increase in frequency of transfer of each strain in the surface mating system.

The synthesis of thin pili was detected using the phage $I\alpha$ or PR64FS.

had been isolated (these insertions were isolated in ColIbdrd-2, which has been found to behave identically to ColIbdrd-1). To extend the range of insertion mutants included in this study, these pCC plasmids were characterized for pilus synthesis along with the pCR and pCI plasmids. The data from these plasmids is given in Table 5.1.2 and the position of these insertions in Figure 5.1.1. The insertions in plasmids pCC17 and pCC24 mapped very close to those in pCR10 and pCR14 in S5 and all of these mutants had identical phenotypes with regard to pilus expression and transfer efficiency. The insertions in pCC11 and pCC25 were mapped close to the insertion in pCR7. All these mutants gave similar results for transfer and pilus expression, but by immune electron microscopy, pCC11 and pCC25 were found to express normal high levels of thick I pili.

Also included in this group is the plasmid pLG264. The insertion in this plasmid was constructed by the recombination method of Winans and Walker (1983), specifically as an insertion in the sog gene (Merryweather et al., 1986b). This plasmid was found to be about 50 fold defective in transfer and expressed both thick and thin I pili normally. As the direction of transcription of the sog gene is now known, it seems unlikely that there is a continuous block of tra genes from S5 to this point. If this were the case and assuming that Tn5 is polar in this system, all the insertions upsteam of this gene would be expected to be at least as transfer defective as pLG264. However the insertions in pCIE8 and pCIE10 are both fully Tra^+ and we know from studies with pCRS1a that the promoter for sog lies between the gene and the insertion in pCIE10 (see Chapter 3). Therefore unlike F, there must be at least two transfer operons.

Three of the Tn5 insertions in the second set of mutants were found to lie in E9, on the other side of the SalI site at the end of S3 to the insertion in pCIE9 (these were the insertions in pCC14, pCC20, pCC26). These plasmids were severely transfer deficient but strains carrying

these plasmids apparently still produced normal thin pili and were shown to produce thick I pili by the ELISA test. When strains carrying these plasmids were screened for thick pili by electron microscopy however, they were found to produce excessive mucoid material and the pili were difficult to prepare (Dr D. Bradley, personal communication). Although only a few thick pili were isolated, it proved impossible to make any quantitative judgement as to the number of pili produced per cell. When the frequency of transfer of these strains was tested in a surface mating, virtually no rescue occurred. Either these mutations affect some fundamental part of the transfer complex of ColIb, or the change in the nature of the cell surface prevents conjugative cell contacts being established. Complementation studies described in Chapter 7 suggest that this latter explanation is probably correct.

The results from the study of these pCC plasmids fit the pattern established by the other transposon mutatants discussed and three blocks of transfer genes now are apparent. The first is in fragment E2 between the insertions in pCR1 and pCR7, the second is from the sog gene in E3 to the insertion in pCIE18 and the third is an undefined region which includes oriT and presumablythe unmapped site of the insertion in pCR5 near this site.

The only plasmids not so far discussed are pCR12 and pCR13. These were two of the primase-defective plasmids isolated using Tn1723 (see Chapter 4). These insertions mutants were found to have only 17 and 27 fold reduced rates of transfer respectively, similar to the transfer deficiency of the primase defective plasmid, pLG250 (Chatfield et al., 1982). The deficiency of pCR12 and pCR13 therefore may be due to their effect on the expression of the sog primase (see Section 5.2). These plasmids produced both thick and thin I pili normally and so seem to delimit the end of the tra region in E2. The effect of all of the plasmids so far described on the expression of the sog gene, is described

in the next section.

5.2 The effects of Tn1723 and Tn5 insertions on the expression of the sog gene.

The ability of sog to suppress the dnaG3 mutation of E. coli has allowed the development of two ways to measure the expression of this gene. Firstly there is the rescue of colony-forming ability at the restrictive temperature for this mutation, as previously described. The second method assays plasmid primase activity in vivo by determining the level of incorporation of ^{3}H-thymidine in to DNA synthesised after the E.coli dnaG primase has been heat inactivated (see Section 2.3.5). Both of these techniques provide quantitative estimates of primase activity (Wilkins et al., 1981).

Before the transposon insertions could be tested for their effect on sog expression, the plasmids had to be introduced in to the strain BW86, which carries the temperature-sensitive dnaG3 lesion. Some of the plasmids were isolated originally in this strain, the others were introduced by transformation in most instances, or by conjugation, in to a nalidixic acid-resistant derivative of BW86, BW86N. (This strain was found to behave identically to the parental BW86 strain when tested at 40°C for colony-forming ability, DNA synthesis and rescue by pCR9.) After the plasmids had been transferred in to these strains, they were tested to check that their mutant phenotype had not altered and that their restriction pattern had not changed.

The results obtained from the two different primase tests are summarised in Table 5.2.1. The results for all the transposon insertion mutants so far mentioned are included, except those in S3 which were all phenotypically Sog^{+} and gave survival coefficients of 0.2 - 0.9 (data not shown). Initially all the plasmids were tested for their effect on

a : DNA synthesis measurements were performed in BW86, at 40°C.

b : See text concerning disparity between colony-forming ability and DNA synthesis figures

Plasmids are arranged in to groups according to the location of the insertion in ColIb in each case.

TABLE 5.2.1 : EXPRESSION OF PRIMASE ACTIVITY BY ColIb DERIVATIVES.

Plasmid in BW86	Colony-forming units per ml. 30°C	40°C	Colony-forming Ability	DNA Synthesis cpm/A_{450}/ml[a]
None	4.9×10^{7}	0	$<2.0 \times 10^{-8}$	12.6
pCR9	5.1×10^{7}	2.6×10^{7}	0.51	410.0
pCR12	1.1×10^{8}	1.1×10^{4}	1.0×10^{-4}	177.0
pCR13	9.0×10^{7}	7.5×10^{3}	8.3×10^{-5}	202.6
pCR1	4.1×10^{7}	0	$<2.4 \times 10^{-8}$	67.6
pCR11	2.0×10^{8}	10	5.0×10^{-8}	39.2
pCR10	1.8×10^{8}	10	5.5×10^{-8}	75.2
pCR14	6.9×10^{7}	1.9×10^{3}	2.7×10^{-5}	ND
pCC17	1.7×10^{8}	8.3×10^{4}	4.8×10^{-4}	51.9
pCC24	1.7×10^{8}	4.1×10^{4}	2.4×10^{-4}	60.1
pCR15[b]	3.6×10^{7}	9.3×10^{3}	2.6×10^{-4}	520.7
pCC11	1.6×10^{8}	1.1×10^{8}	6.1×10^{-1}	ND
pCC25	2.5×10^{8}	2.7×10^{8}	1.1	ND
pCR3	3.0×10^{7}	5.8×10^{5}	1.9×10^{-2}	447.0
pCR4	6.7×10^{7}	6.4×10^{6}	9.6×10^{-2}	448.4
pCR6	4.0×10^{7}	2.0×10^{7}	0.51	ND
pCR7	2.2×10^{7}	1.2×10^{6}	5.3×10^{-2}	396.9
pCIE8	1.9×10^{8}	5.2×10^{7}	0.27	ND
pCIE10	1.8×10^{8}	7.9×10^{7}	0.44	ND
pLG264	4.5×10^{7}	4.9×10^{7}	1.1	354.6
pCIE18	2.8×10^{7}	3.7	1.3×10^{-7}	ND
pCC14	1.4×10^{8}	1.6×10^{8}	1.1	311.7
pCC20	1.3×10^{8}	1.6×10^{8}	1.2	362.9
pCC26	1.7×10^{8}	1.7×10^{8}	1.0	416.4

colony-forming ability at 40°C. When this was done it was again found that insertions that had similar locations had similar effects on sog expression.

The most severe effects on sog expression were produced by the group of insertions in S5 (the insertions in pCR1, pCR10 and pCR11) with the insertions to the left of these, in pCR14, pCC17 and pCC24, having slightly less effect on colony-forming ability at 40°C, but giving the same effect on DNA synthesis. Next to this group of insertions lies the insertion in pCR15. The strain containing this plasmid gave conflicting data for the two measurements. Its colony forming ability was low and yet it was capable of normal sog$^+$ levels of DNA synthesis, suggesting that the sog gene must be being expressed normally. To try and clarify this matter, the effect of the plasmid on the colony-forming ability of the dnaG$^+$ strain BW85 at high temperature was tested. When this was done, it was found that the viability of this strain at high temperature was also reduced (9.7×10^7 cfu/ml at 30°C, 7.1×10^6 cfu/ml at 40°C; colony-forming ability = 7.3×10^{-2}) and even at the normal growth temperature of 37°C, colonies formed were very small and growth rate in liquid culture was slow. The reduced viability, taken with a low rescue figure (rescue by ColIb sog$^+$ plasmids can vary between 0.2 and 1.0), would produce the apparent deficiency in sog expression of pCR15. Therefore it was concluded that the insertion in pCR15 has no effect on sog primase expression. Knowing that the mutation in pCR15 disrupts pilus assembly provides a possible expanation for the reduction in viability. As was discussed in Chapter 1, expression of sex pili makes the host cell more fragile. Possibly unassembled pilin or residual assembly complexes in the cell membrane may affect the colony-forming ability of the cells carrying this plasmid.

All of the other plasmids with insertions between S5 and the sog gene (pCC11 to pLG264) were phenotypically Primase$^+$, as determined by both

sets of measurements. This indicates that whatever the effect the insertions in S5 exert on the expression of the sog gene, it cannot be due to polarity. The effect of the insertions in S5 now appear to be more pleiotropic than was at first apparent. They cause decreased levels of thick pilus synthesis, abolish thin pilus synthesis and reduce primase activity to within five fold of that given by the BW86 control at the non-permissive temperature. Thus they seem to be very similar to the insertion in E18, which was also shown to be Sog$^-$ from colony-forming ablility measurements (again this cannot be a polar effect since the direction of transcription of the sog gene is towards the insertion in pCIE18). It begins to appear that the ColIb plasmid has a two part regulatory system and that for full expression of all the transfer genes, both the S5 and E18 regions are required. Further evidence for this model is described in Section 5.3 and in Chapters 6 and 7.

The insertions in S7, in plasmids pCR12 and pCR13, represent somewhat of an anomaly. They were initially isolated by their Sog$^-$ phenotype in colony-forming ability tests and yet, as shown by the DNA synthesis data, they retained an intermediary level of primase activity (approximately half that of the control sog$^+$ values). Again they cannot exert a polar effect on the expression of the sog gene. These plasmids were found to score positively for all other tra related phenotypes, so they cannot be part of the positive regulatory system either. When the sog gene was cloned in pBR325 (to give pLG215), it was found that despite increased gene dosage the cloned gene could not complement the dnaG mutation as well as the gene expressed from the parental plasmid (Chatfield et al., 1982). In an attempt to explain this phenomenon, a sogB determinant was proposed, also encoded by ColIb, which would allow the ColIb primase to interact with the host cell primosome more efficiently than the enzyme could alone (Chatfield, 1985). The insertions in S7 may not affect the sog gene directly, but may represent a mutation in the sogB determinant

which reduces the ability of sog primase to suppress the dnaG mutation of the host cell.

5.3 The effect of Tn5 in pLG264.

The Tn5 insertion in pLG264 was constructed by A. Merryweather, using the recombination method of Winans and Walker (1983). Insertion of Tn5 introduces a transcriptional terminator into a gene and therefore truncated polypeptide products may be produced corresponding to the region of the gene that is promoter-proximal to the site of the insertion (Berg et al., 1980). In a collaborative study with A. Merryweather, the polypeptide products of the sog gene from pLG264 were identified.

As shown in Figure 5.3.1, the insertion was originally generated in a cloned E3 fragment and was mapped to 2.5 kb from the left end of E3. The sog gene specifies two polypeptides of 240 Kd and 180 Kd (Wilkins et al., 1981) and the insertion site corresponded to the C-terminal region of the sog polypeptides (Merryweather et al., 1986b). The transposon-induced mutation was then recombined into ColIbdrd-2 to produce pLG264. This plasmid was phenotypically Sog^+ however, because only the N-terminal third of the 240 Kd polypeptide expressed from this gene is required for primase activity. Using antiserum raised against sog polypeptides (Wilkins et al., 1981) it was possible to identify the effect of the Tn5 insertion on the products of this gene by Western blot analysis.

Total protein from a crude cell extract was separated on a 10% SDS PAGE gel. The proteins were transferred to a nylon membrane and incubated with rabbit anti-sog polypeptide antiserum. Specific binding of this antibody to the sog polypeptides was amplified using secondary and tertiary antibodies and a colour reaction developed using hydrogen peroxide and 4-chloro-1-naphthol (see Section 2.13). Figure 5.3.2 shows the results of such an experiment. Lane 1 is protein from the BW86 host

FIGURE 5.3.1 : Map of pLG262.

This map is based on that given by A. Merryweather (1986). Restriction sites are given for BglII (Bg), EcoRI (E), HindIII (H) and SalI (S). The plasmid pLG262 consists of the E3 fragment of ColIb cloned into pBR325, into which Tn5 has been inserted at the site shown. The arrows indicate the coding region and direction of transcription of the 240 Kd and 180 Kd polypeptide products of the sog gene. The orientation of Tn5 is indicated by the relative position of IS50L (L) and IS50R (R); see Figure 4.3.1.

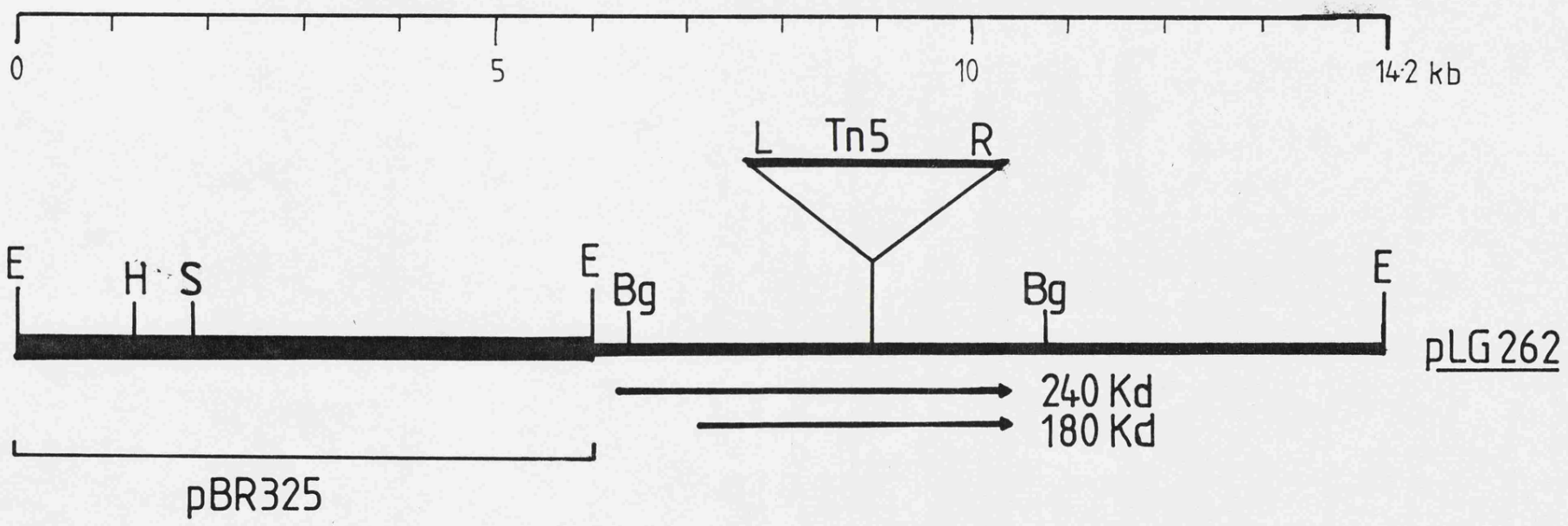
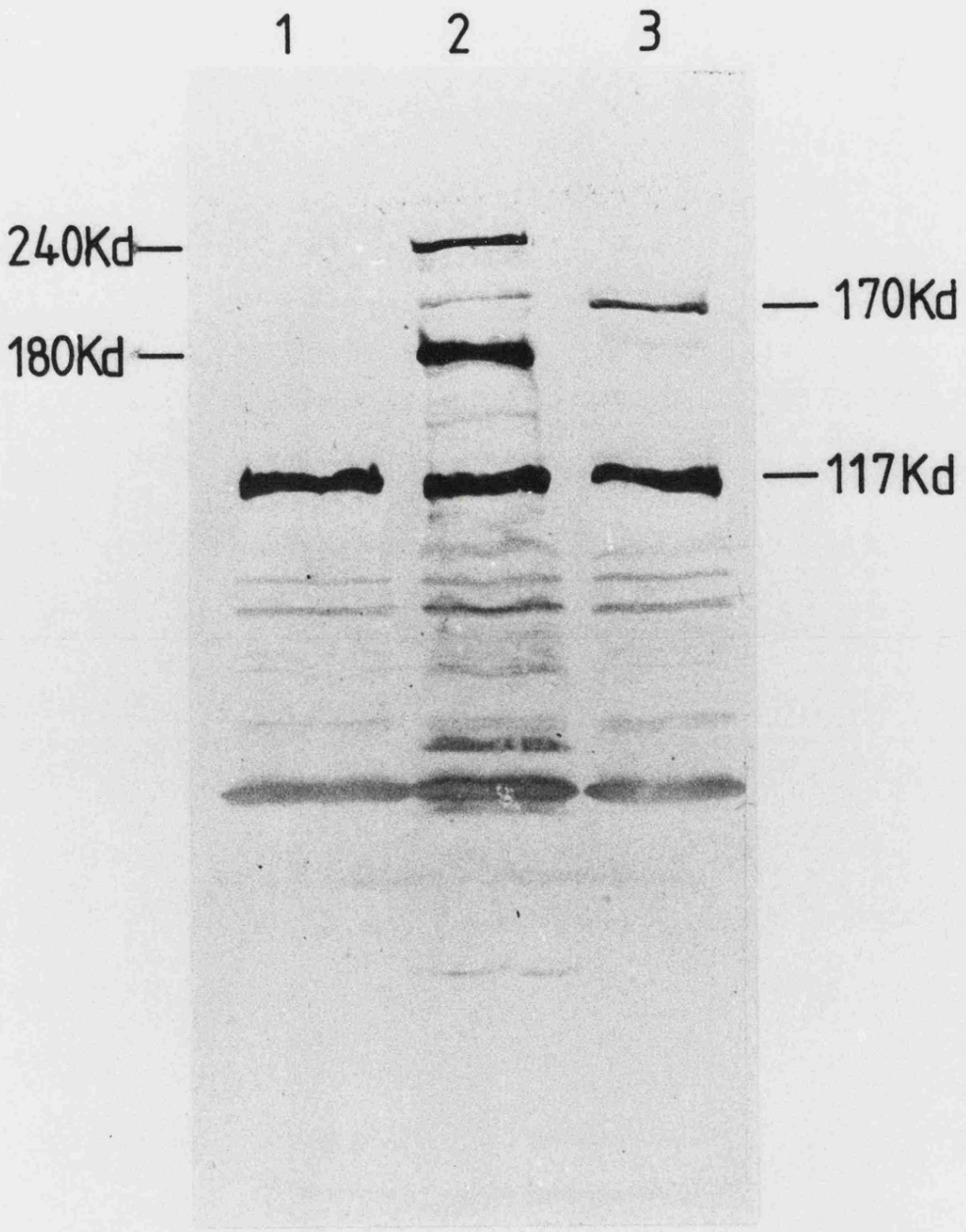

0
5
10
14·2 kb
L
Tn5
R
E
H
S
E
Bg
Bg
E
pLG 262
240 Kd
180 Kd
pBR325

FIGURE 5.3.2 : Detection of sog polypeptides by Western blot.

Lane	Strain	sog polypeptides.
1	BW86	-
2	BW86(pLG269)	240 Kd, 180 Kd
3	BW86(pLG264)	170 Kd

pLG269 is a ColIb*drd-2*::Tn*5* Tra^+ control strain; *sog* polypeptides were detected after transfer of proteins from an SDS-PAGE gel on to a nitrocellulose filter and exposing to anti-*sog* polypeptide antibody (see Section 2.13). The 240 Kd and 180 Kd polypeptides detected from BW86(pLG269) are indicated on the left of the gel, and the 170 Kd truncate detected from BW86(pLG264) on the right. Also marked is the 117 Kd *E.coli* polypeptide which corresponds to the predicted size of the 180 Kd polypeptide truncate (240 Kd and 180 Kd polypeptides were estimated as 208 Kd and 150 Kd from this gel; see text).


1
2
3
240Kd
180Kd
170Kd
117Kd

strain illustrating the background cross-reaction that occurs with this antiserum. In lane 2 the bands corresponding to the 240 Kd and 180 Kd polypeptides are marked. Lane 3 contains total protein prepared from a strain carrying pLG264 and the 170 Kd polypeptide marked corresponds to the predicted size of the truncated 240 Kd polypeptide. The apparent molecular weight of the smaller sog polypeptide was estimated as 150 Kd from these gels, but for historical reasons will be referred to as the 180 Kd polypeptide (see Wilkins et al., 1981 and Merryweather et al., 1986b). The second predicted truncated polypeptide was not detected. It is possible that the truncate was unstable and therefore degraded or that it comigrated with the 117 Kd E.coli protein that cross-reacted with the antiserum and is not seen.

It has been shown by complementation studies that the transfer deficiency of pGL264 is the result of the disruption of the 180 Kd polypeptide and a role has been proposed for this protein in the transfer of the single-stranded DNA between conjugating cells (Merryweather et al., 1986b). It was also shown that no transfer genes lie on the promoter distal side of the sog gene in the same transcriptional unit. However this does not exclude the possibility that tra genes may lie between the end of this transcriptional unit and E18.

5.4 Complementation of pCRS1a by pCIE18 and pCR1.

In an attempt to resolve whether the insertions in E18 and S5 did affect controlling elements, the plasmid pCRS1a containing the cloned sog gene with its promoter (see Figure 3.2.1) was introduced into the strains BW86(pCIE18) and BW86(pCR1) by transformation. These combinations of plasmids were tested for their ability to suppress the DnaG$^-$ phenotype of the host strain (data presented in Table 5.4.1). As can be seen, neither pCIE18 nor pCR1 could supply the positively required trans-acting

TABLE 5.4.1 : AFFECT OF pCR1 AND pCIE18 ON THE EXPRESSION OF sog FROM pCRS1a.

Plasmid(s) in BW86	Colony-forming units per ml. 30°C	40°C	Colony-forming Ability
pCRS1a	2.4×10^{7}	0	$<2.4 \times 10^{-7}$
pCRS1a,pCIE18	$1.0 \times x10^{8}$	40	3.9×10^{-7}
pCRS1a,pCR1	1.5×10^{8}	20	1.4×10^{-7}
pCRS1a,pLG250[a]	1.2×10^{8}	5.9×10^{6}	4.9×10^{-2}
pCRS1a,pLG250[b]	9.9×10^{7}	5.2×10^{6}	5.3×10^{-2}

a : Constructed with pCRS1a DNA from BW86(pCRS1a,pCIE18).

b : Constructed with pCRS1a DNA from BW86(pCRS1a,pCR1).

Expression of the sog gene was determined by plating cells on non-selective L-agar to give single colonies at both the permissive (30°C) and non-permissive temperatures (40°C) for the dnaG3 lesion in BW86. Rescue of colony-forming ability at 40°C is expressed as the ratio of colonies formed at 40°C to the number formed at 30°C (colony-forming ability).

factor needed for the expression of sog from pCRS1a (see Chapter 3). To check that pCRS1a was not defective in some way and therefore could no longer express plasmid primase activity, plasmid DNA was prepared from these strains and used to transform BW86(pLG250). Ampicillin-resistant transformants were tested again for their ability to grow at the restrictive temperature for the chromosomal dnaG mutation and were all found to do so, implying that the pCRS1a sog gene was still functionally intact.

This provides fairly substantial evidence that S5 and E18 both contain positive control elements, although it does not preclude the possibility that one of these may represent an operator site for a trans-acting regulator. This could not be a conventional operator site at the beginning of the transcriptional unit however, as neither of these sites are near the promoter of the sog gene. Studies of the effect of the insertions in these plasmids on other coordinately regulated conjugation genes (see Chapter 6) support the model for a two part regulation system.

5.5 Summary.

Using the I_1 male-specific phage, it has been possible to identify a region between coordinates 67.0 and 82.0 which is required for the synthesis of the thin I_1-pilus. Insertions in this region do not seem to disrupt thick I_1-pilus assembly. Therefore it may be proposed that the two types of pilus do not share one assembly mechanism and that the genes required for thick pilus synthesis are located in a different region of the plasmid.

The effect of the insertions on the expression of the sog gene has led to the identification of at least two tra regions (from coordinates 67.0 to 82.0 and 57.0 to 42.0) and has demonstrated that at least two

independent regions supply *trans*-acting factors neccesary for the expression of this gene and of the other *tra* genes. Further evidence for this model is described in Chapter 6.

CHAPTER SIX : Location of the nuclease and exclusion determinants.

Introduction

The only remaining transfer-related functions identified for ColIb are the nuclease gene (*nuc*) and the exclusion determinant (*eex*). The presence of a plasmid-encoded nuclease activity was first detected when a phenol-lysis method (Klein *et al.*, 1980) was used to isolate plasmids from strains carrying ColIb and ColIb*drd-1*. It was found that DNA from the derepressed strain was degraded, and instead of a discrete plasmid band, the DNA formed a smear of decreasing molecular weight (Wilkins, unpublished data; see Figure 6.1.1). ColIb-encoded nuclease is apparently similar to that identified from the IncN plasmid pKM101 (Winans and Walker, 1983; see Chapter 1). Even though the pKM101 nuclease gene was found to lie between two blocks of transfer genes, this gene was not coordinately expressed with them. Unlike this, the ColIb nuclease gene is only expressed from *drd* plasmids, but the physical location of the gene was not known.

The exclusion determinant of ColIb (*eex*) on the other hand is expressed independently of the other transfer functions (this work), but still forms an important part of the conjugation system as described in Chapter 1. By analogy with the F, N and P conjugation systems, the ColIb exclusion determinant was expected to lie near or within other blocks of conjugation genes. It was therefore of interest to see what effect, if any, the insertions in this region had on transfer. The location of *eex* was determined by Chatfield *et al.* (1982) when this determinant was cloned on an 3.4 kb *Eco*RI restriction fragment that mapped to coordinates 37.0 and 42.0 on the map of ColIb. It is feasible that the ColIb nuclease gene may also play some role in exclusion and therefore mutations which affected nuclease expression were also tested for their ability to exclude ColIb plasmids.

6.1 Expression and role of the nuclease gene.

Activity of the nuclease gene was assayed by preparing plasmid DNA by a phenol-lysis method (see Section 2.3.6). If the gene was being expressed, the nucleolytic activity caused the degradation of both plasmid and chromosomal DNA (see Figure 6.1.1). All the transposon insertions were tested for this activity and the results for representative members of each group are summarised in Table 6.1.1.

Once again the insertions in S5 and E18 are seen to be exerting a pleiotropic effect over the expression of another of the coordinately controlled conjugation genes. However this time it is the insertions in S5 that seem to exert an absolute effect over the expression of the nuclease gene, whereas the insertion in E18 only diminished nuclease activity (compare this with the effects of these two insertions on thick I pilus expression). This result again agrees with the model of there being two factors which have an interrelated role in the control of expression of the conjugation genes of ColIb.

The only other insertion that abolished nuclease activity was that in E8. The location of this insertion takes on more significance when it is found that this fragment is involved in the rearrangements of ColIb DNA comparable to those described by Komano *et al.* (1986) in R64. When the pCIE8 plasmids were isolated, the new restriction fragment corresponding to E8 and the Tn*5* DNA, was found to come in one of two forms, the difference in size of which was the same as the difference in size of the two forms of E8 which are normally found in equimolar quantities (see Figure 4.3.5A). It seems that once Tn*5* was inserted in to the fragment, the invertible region became fixed in one orientation. On closer examination, *Eco*RI digests of both pCR1 and pCIE18 seemed to have the E8 fragment fixed in predominantly one orientation or the other (see Figures 4.2.2 and 4.3.5B, lanes 2 and 4 respectively). So it seems that

FIGURE 6.1.1 : Nuclease assay.

Agarose gel showing the effect of the expression of the nuclease gene on ColIb DNA prepared by the method of Klein et al. (1980); see Section 2.3.6.

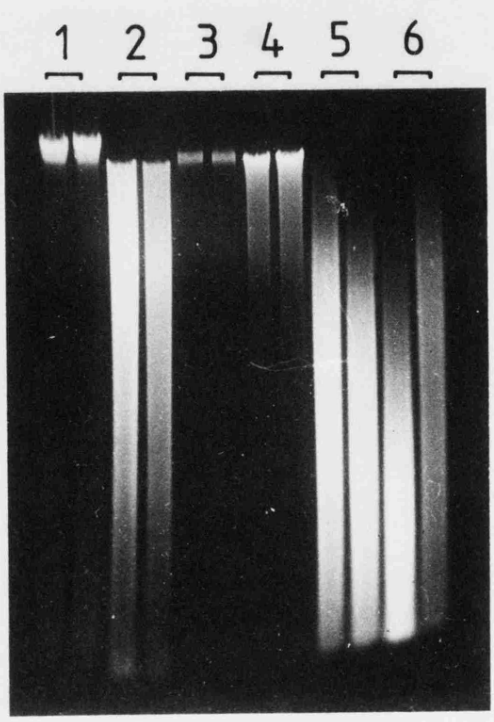
Lane	Strain
1	Bw85
2	BW85(pCIE10)
3	BW85(pCIE8)
4	BW85(pCIE18)
5	BW85(pCR7)
6	BW85(pCR9)

Each strain was tested in duplicate. Samples of DNA run on a 0.7% (w/v) agarose gel at 75 V for 4 hours.

TABLE 6.1.1 : NUCLEASE ACTIVITY OF TRANSPOSON INSERTION MUTANTS OF ColIb

Strain	Nuclease Activity
BW85	-
BW85(pCR9)	+
BW85(pCR1)	-
BW85(pCR10)	-
BW85(pCR15)	+
BW85(pCC11)	+
BW85(pCR3)	+
BW85(pCR7)	+
BW85(pCIE8)	-
BW85(pCIE10)	+
BW85(pLG264)	+
BW85(pCIE18)	+/-
BW85(pCC14)	+
BW85(pCR5)	+
BW85(pCIE14)	+

FIGURE 6.1.1



abolishing nuclease activity also destroys the ability of the inversion region to rearrange.

Other well characterised DNA inversion include the G segment of Mu and C segment of P1 causing a change in the host range of these phage (Kutsukake and Iino, 1980; Gipphart-Gasler et al., 1982; Hoess et al., 1982) and phase variation of fimbriae expression in Neisseria gonorrhoeae, E.coli and Salmonella typhimurium (Hagblom et al., 1985; Freitag et al., 1985; Enomoto et al., 1983; Bruist and Simon, 1984; see also review by Craig, 1985). These rearrangements have been found to involve the inversion of a single invertible segment of DNA which can be in one of two orientations. Most of these inversions have been found to cause variation of surface structures. The site-specific recombinases that mediate these rearrangements have been found be related, and also related to the site-specific resolvase (the product of tnpR) of Tn3. Mg^{2+} is an essential co-factor for these recombinases, and in the absence of this cofactor the tnpR product has an endonucleolytic activity, whilst addition of 10 mM Mg^{2+} allows the recombination event to proceed through this cleavage product to produce two resolved circular molecules. A site-specific recombinase that mediates either inversions or deletions of the IncN plasmid R46 has also been identified (Dodd and Bennett, 1983). The recombinase in the latter system has been shown to be interchangeable with the resolvase of TnA (Nash, 1980) and can utilise the res site of an inserted TnA transposon and a res analogue (the per site) in the plasmid genome, which lies between the end of the tra region and the arsenate-resistance determinant. The plasmid-encoded recombinase gene (per) is found within 2 kb of the per site (Dodd and Bennett, 1986) and apparently has a role in stable maintenance of the plasmid, resolving dimers and ensuring efficient partitioning.

The site-specific recombinase (rci) of R64 was located on the same EcoRI fragment that was involved in the inversion event. It may be that

the nuclease gene of ColIb and the rci gene of R64 are the same gene. Certainly the insertion in the invertible fragment of ColIb abolishes both the DNA rearrangement and the nuclease-mediated DNA degradation, but this hypothesis will require further investigation. What is of interest is that if, as suggested by Kamano et al. (1986), this inversion controls the expression of a very rare event, the location of this gene at the end of a region known to be involved in the expression of a ColIb-specific surface structure, the thin pilus, may have a new significance.

6.2 Location of the exclusion determinant.

To measure exclusion, a system had to be derived that would overcome the problem of incompatibility of the two plasmids. For instance it would be impossible to differentiate between a mating with an eex^+ recipient where the donor plasmid is excluded, and a mating where the incoming plasmid was incompatible with the resident plasmid. To avoid this complication, the exclusion is measured by the frequency of mobilization of an oriT recombinant plasmid by a ColIb donor strain. The transferred oriT plasmid is compatible with the resident plasmid and therefore any deficiency in its frequency of transfer is caused by exclusion directed against the conjugation system of the mobilizing plasmid.

The donor strain used in this test was BW103(pLG221,pLG2009). The recA background of this strain reduces the possibility of transferring pLG2009 as a cointegrate with pLG221 following a homologous recombination event. The donor strain was mated with either BW97 or BW86N recipients which carried the ColIb derivatives, selecting for transconjugants with ampicillin and nalidixic acid. The exclusion index was determined as the ratio between the number of oriT plasmids transferred into the recipient strain without and with the test plasmid. The results of these tests are

TABLE 6.2.1 : EXCLUSION BY TRANSPOSON INSERTION MUTANTS OF ColIb.

Plasmid in recipient strain[a]	Exclusion Index[b]
None	1.0[c]
ColIb	1.3×10^3
ColIb*drd-1*	1.2×10^4
pCR9	1.3×10^3
pCR1	2.4×10^4
pCR15	7.8×10^2
pCR7	3.5×10^2
pCIE8	3.9×10^3
pCIE10	3.6×10^3
pLG264	1.3×10^3
pCIE18	1.8×10^2
pCC14	1.9×10^3
pCC20	1.7×10^3
pCC26	1.7×10^3
pCIE9	3.8×10^3
pCIE15	1.5×10^2

a: Recipient strains were either BW97 or Bw86N.

b: The exclusion index represents the frequency of transfer of *oriT* plasmids into a plasmid-free recipient / the frequency of transfer of *oriT* plasmids into a plasmid-carrying recipient.

c: 3.9×10^6 Nal^R Ap^R transconjugants per ml, per hour.

summarised in Table 6.2.1.

From these results it can be seen that all the transposon mutants expressed surface exclusion. A great degree of variability existed in the number of transconjugants obtained in each experiment and the figures presented represent an average number reached after each plasmid had been tested several times. Therefore it is difficult to make any conclusions from the data where only a ten fold difference exists in the level of exclusion. What can be deduced is that none of the insertions directly affect the expression of the ColIb exclusion system, and also that those plasmids defective in nuclease expression (pCIE8, pCIE18 and insertions in S5) do not show consistently low exclusion indices, ruling out an involvement of the *nuc* gene in exclusion.

The location of the *eex* determinant of ColIb within the 3.4 kb *Eco*RI fragment on which it is cloned was unknown, although by comparison with the cloned exclusion determinant of R144, it was presumed to lie near the *Hin*dIII and *Sal*I sites of E9 (see Figure 1.5). The position of the Tn*5* insertion in pCIE9 would be 0.4 kb beyond the predicted position of the end of the gene. The exact site of the other Tn*5* insertions in E9 (in plasmids pCC14, pCC20 and pCC26) was unknown, except that they were on the opposite side of the *Sal*I site to the insertion in pCIE9. To locate the sites of these insertions more exactly, the E9 fragments from these plasmids were cloned into the vector pBR328. A shot-gun cloning technique was used, transformants being selected which carried both the vector markers and the kanamycin resistance determinant of Tn*5*. Using this method the E9 fragments from pCC14 and pCC20 were both cloned and the plasmids were called pE9-14 and pE9-20 respectively (a second *Eco*RI fragment of 1.05 kb was also contained in pE9-20 along with the E9 fragment, see Figure 6.2.1). The corresponding fragment from pCC26 failed to be cloned despite repeated attempts. Restriction data for pE9-14 and pE9-20 and the site of the insertion in each case is shown in

FIGURE 6.2.1

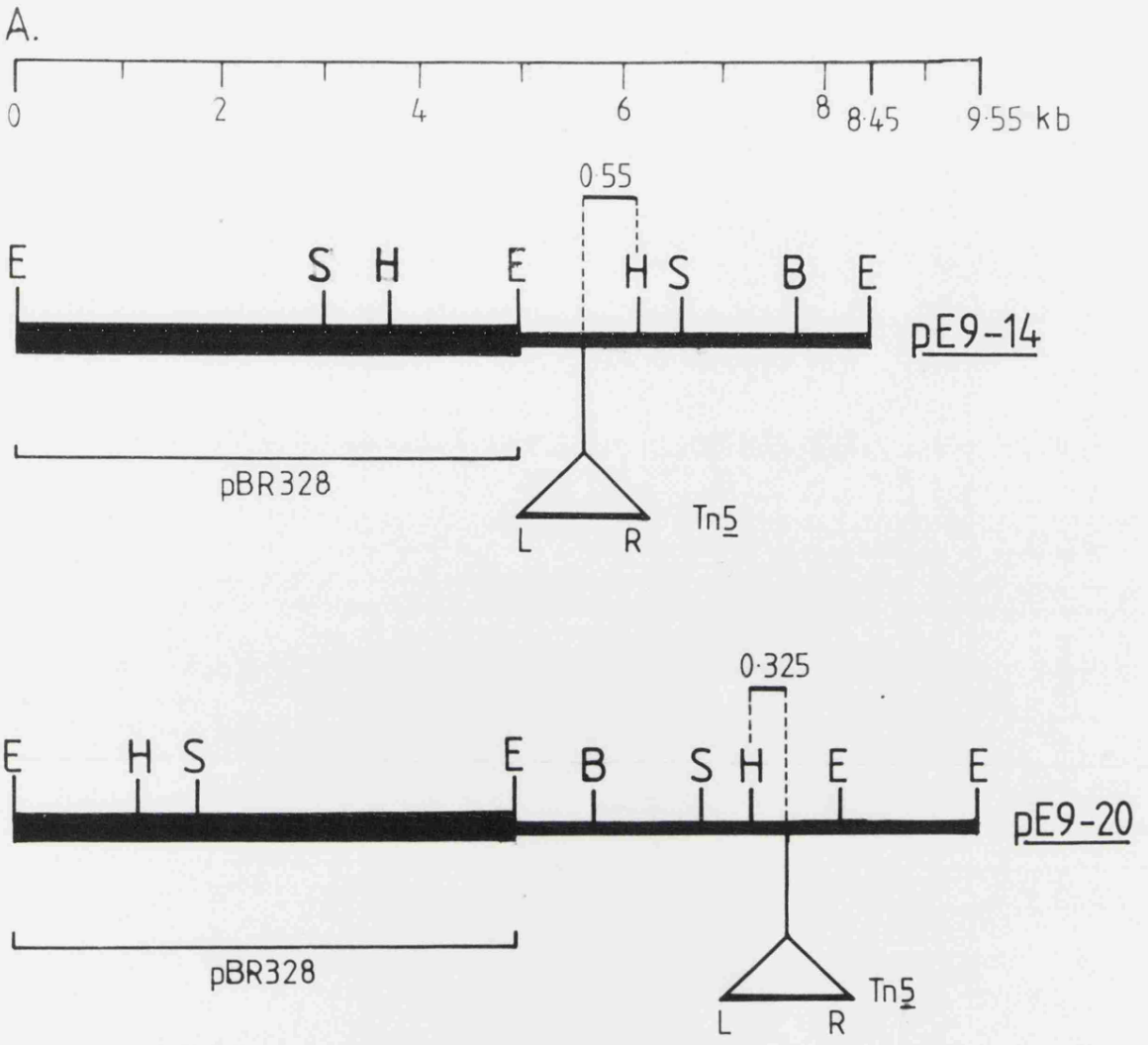
A. Restriction maps of pE9-14 and pE9-20.

These maps are based on the map of the cloned E9 fragment in pLG252 (Merryweather, 1986). Restriction sites are indicated for BglII (B), EcoRI (E) and SalI (S). The site of the Tn5 insertion and its distance from the HindIII site in E9 is shown in each case. Tn5 is orientated by the relative position of IS50L (L) and IS50R (R); see Figure 4.3.1.

B. Agarose gels of restriction digests of pE9-14 and pE9-20.

Lane	DNA	Enyzme	Restriction fragments (kb)				Total
1	pE9-14	HindIII	6.03	3.35	2.95	1.55	13.85
2	pE9-14	SalI	5.01	4.95	4.00		13.96
3	pE9-14	BglII	7.80	3.55	2.45		13.80
4	pE9-20	HindIII	5.94	4.17	3.55	1.38	14.94
5	pE9-20	SalI	6.80	4.85	3.31		14.96
6	pE9-20	BglII	9.10	3.35	2.45		14.90

The second gel shows the two plasmids cut with EcoRI. The bands corresponding to pBR328, E9::Tn5 and the extra 1.05 kb EcoRI fragment in pE9-20 are marked. Both gels are 0.8% (w/v) agarose, run at 25 V for 16 hours.


A.
0
2
4
6
8
8·45
9·55 kb
0·55
E
S
H
E
H
S
B
E
pE9-14
pBR328
Tn5
L
R
0·325
E
H
S
E
B
S
H
E
E
pE9-20
pBR328
Tn5
L
R

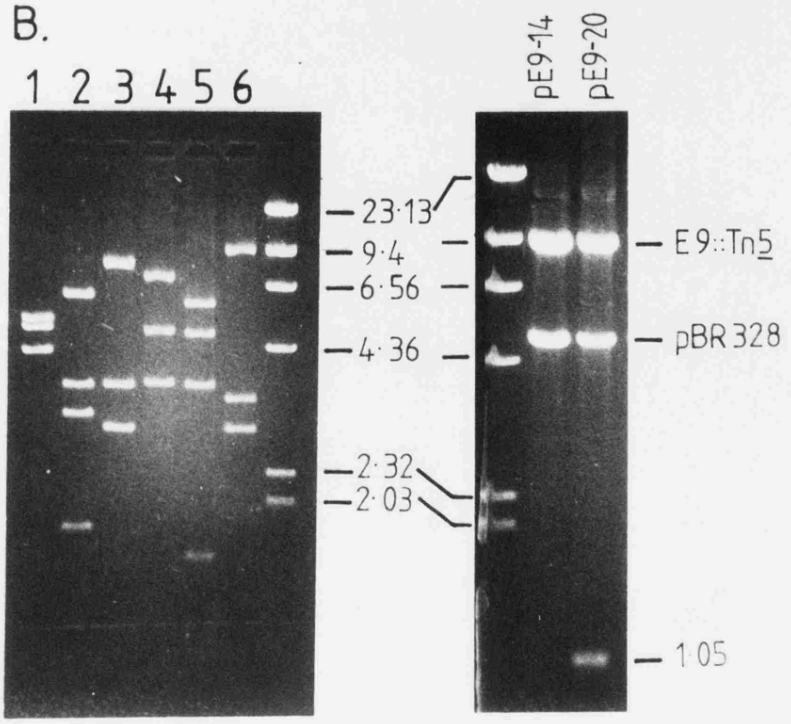

B.
1 2 3 4 5 6
pE9-14
pE9-20
23·13
9·4
6·56
4·36
2·32
2·03
E9::Tn5
pBR328
1·05

Figure 6.2.1, (these maps are based on the restriction map of the cloned E9 fragment of ColIb, pLG252; Merryweather, 1986).

Both of these Tn*5* insertions are just upstream of the proposed location of the *eex* determinant (0.3 kb in pE9-14 and 0.13 kb in pE9-20). Taking these results with the position of the insertion in pCIE9 (as indicated on the map of pLG252, Figure 6.2.2), it would seem that if the ColIb *eex* determinant is in an analogous position to that of R144, then like R144 and but unlike F and RP4, the exclusion determinant is not located close to any other transfer genes.

In a final analysis of this region, the exclusion ability of these plasmids was determined, and also the exclusion index of two recombinant plasmids carrying the two halves of E9 separately on two ColIb *SalI* fragments (pCRS3 and pCRS6; the region covered by each of these plasmids is marked in Figure 6.2.2). Because all these recombinant plasmids carried ampicillin-resistance determinants, it was necessary to use different *oriT* recombinant plasmids in the exclusion test. For the pE9 plasmids and pLG252 the strain used as donor was BW103(pLG221,pGSS33). pGSS33 is a recombinant plasmid based on R300B (IncQ) and pBR322 which is efficiently mobilized by $IncI_1$ plamids (Sharpe, 1984). For pCRS3 and pCRS6 the donor strain was C600(ColIb*drd-1*,pTB92) where the mobilizable plasmid is a Km^R derivative of R300B (Barth *et al.*, 1981). The results are summarised in Table 6.2.2.

The results show that both pE9-14 and pE9-20 retained the ability to express the exclusion determinant. The two cloned fragments are inserted in to the vector in opposite orientations, and the E9 fragment in pE9-14 is in the opposite orientation, with respect to the vector, to the E9 fragment in pLG252. Therefore the exclusion gene(s) must be expressed from their own promoter on the fragment. Neither of the plasmids carrying the two halves of E9 expressed exclusion, therefore the gene must span the *SalI* site at coordinate 7.6 in E9 and its position is

TABLE 6.2.2 : EXCLUSION BY RECOMBINANT PLAMSIDS OF ColIb.

Plasmid in Recipient Strain[a]	Exclusion Index[b]
None	1.0[c]
pLG252	1.4×10^3
pE9-14	1.2×10^2
pE9-20	1.2×10^2
pCRS3	1.3
pCRS6	1.8

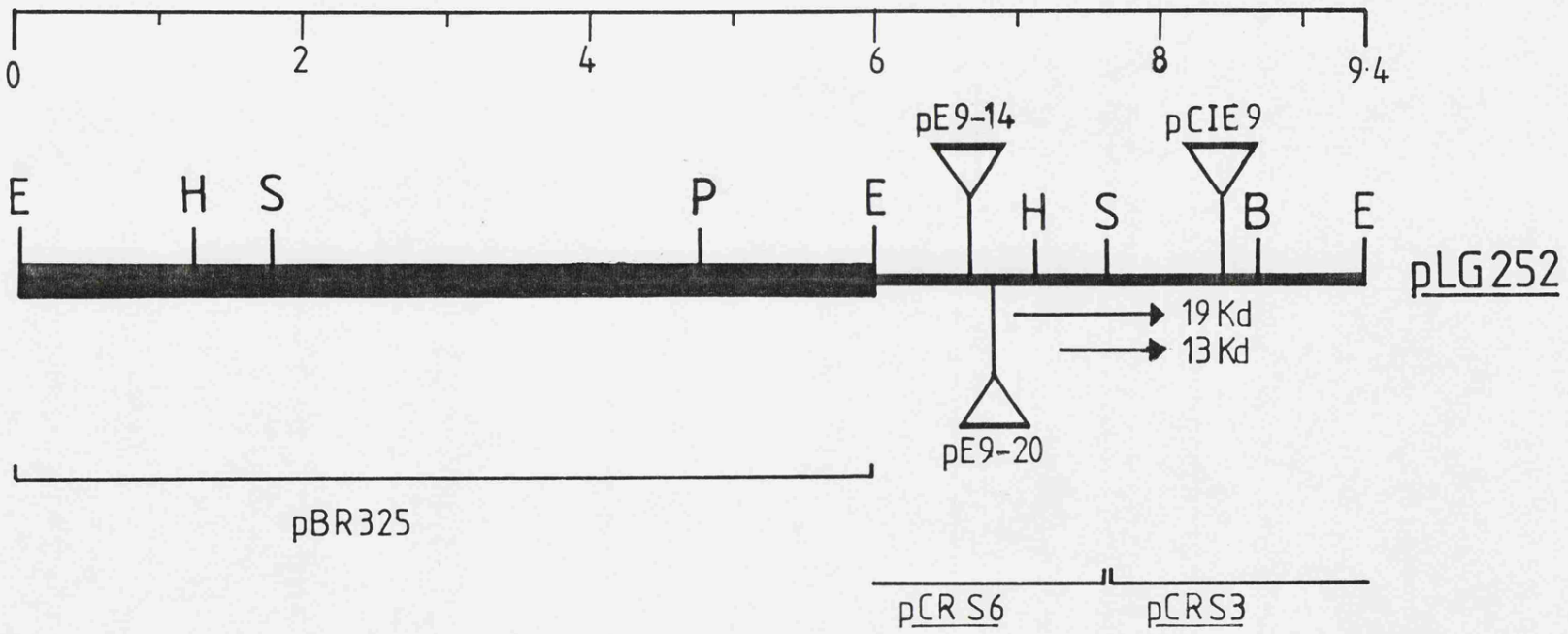
a: Host strains were either BW97 or Bw86N.

b: The exclusion index represents the frequency of transfer of oriT plasmids into a plasmid-free recipient / the frequency of transfer of oriT plasmids into a plasmid-carrying recipient.

c: 1.6×10^6 Nal^R Cm^R transconjugants per ml per hour.

FIGURE 6.2.2 : Location of the eex gene of ColIb.

The map of pLG252 shows the sites of the insertions of the transposons in pE9-14, pE9-20 and pCIE9. Restriction sites are shown for BglII (B), EcoRI (E), HindIII (H) and SalI (S). The arrows represent the predicted position and direction of transcription of the 19 Kd and 13 Kd exclusion polypeptides identified from R144. Also marked are the regions of E9 contained in the plasmids pSRS3 and pCRS6.


0
2
4
6
8
9·4
pE9-14
pCIE9
E
H
S
P
E
H
S
B
E
pLG252
19 Kd
13 Kd
pE9-20
pBR325
pCR S6
pCRS3

delimited by the transposon insertions close to both sides of this site (see Figure 6.2.2). Thus the position of the eex determinant on ColIb, predicted by comparison with the restriction map of R144 in this region, is correct.

6.3 Summary.

The location of both the nuclease and the entry exclusion genes has been determined. Inactivation of the ColIb-encoded nuclease also seems to abolish rearrangement in the inversion region at coordinates 66.0 to 67.0, which suggests that these two functions may be related. As inactivation of the nuclease gene, which is coordinately regulated with the conjugation genes, has no apparent effect on the transfer frequency or exclusion of pCIE8, no role can be assigned to this gene to date. The effect of the insertions in the two putative control regions of ColIb (S5 and E18) on nuclease expression lends support to the model of a two part regulatory system, the components of which affect the level of transcription of different transfer functions to different extents.

The eex gene has been shown to be expressed independently of the other conjugation genes, from a promoter within the E9 fragment. By analogy with the R144 exclusion determinant, the ColIb eex gene is expected to be transcribed in the same direction as the sog gene, towards the oriT site.

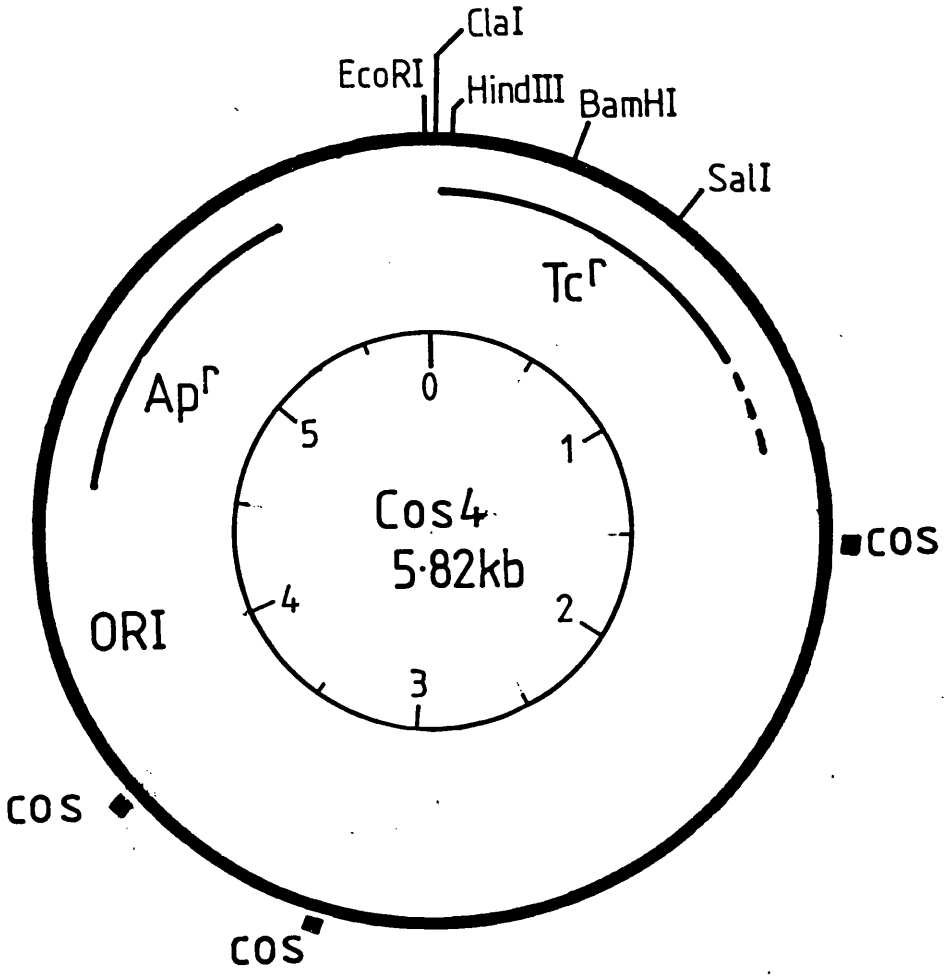
CHAPTER SEVEN : Construction and characterisation of cosmid clones of ColIbdrd-1.

Introduction

The results of the transposon insertion analysis indicated that the region of ColIb required for thin I pilus synthesis was between coordinates 67.0 and 82.0. To try and dissect the genes involved in this process, it was decided to isolate a series of cosmid clones from this region that could express the thin pilus independently of the rest of the ColIb conjugation system. Use of a cosmid vector for this purpose had three advantages. Firstly, the size of the fragments inserted into the vector are exceptionally large (the insertion of ~45 kb of DNA in to the vector is required to allow packaging of the recombinant DNA in to the phage heads) and this should allow the large number of genes predicted to be necessary for pilus synthesis to be isolated. Secondly, the use of the BamH1 restriction site in the vector allowed the use of Sau3A to generate random fragments of ColIbdrd-1 by partial digestion, overcoming the problem of a lack of suitable restriction sites in the ColIb transfer region. Thirdly, once packaged in to phage heads, the recombinant molecules are easily introduced into host strains by infection, avoiding the problem of low transformation frequencies of large DNA molecules. It was hoped that by isolating a series of cloned fragments with varying end-points from the region involved in thin pilus syngthesis, that it would be possible to delimit the minimum region essential for thin pilus expression.

The cosmid clones would also be compatible with ColIb and therefore could be used in conjunction with the various insertion mutants of ColIb in complementation tests to help identifiy the transcriptional organisation of the transfer genes.

FIGURE 7.1.1 : A map of Cos4.



This map of Cos4 is based on unpublished data from Lehrach and Reedy (see Section 2.7). Relevant restriction sites near the BamHI site used in the cloning procedure are shown, along with the location of the resistance determinants (Ap^R = ampicillin-resistance, Tc^R = tetracycline-resistance). Also shown are the plasmid origin of replication (ORI) and the cos sites (represented by the boxes) required for packaging of recombinant DNA in to the phage heads. The distance between the cos sites must be ~45 kb after insertion of foreign DNA in to one of the resistance determinants to allow succesessful packaging of DNA.

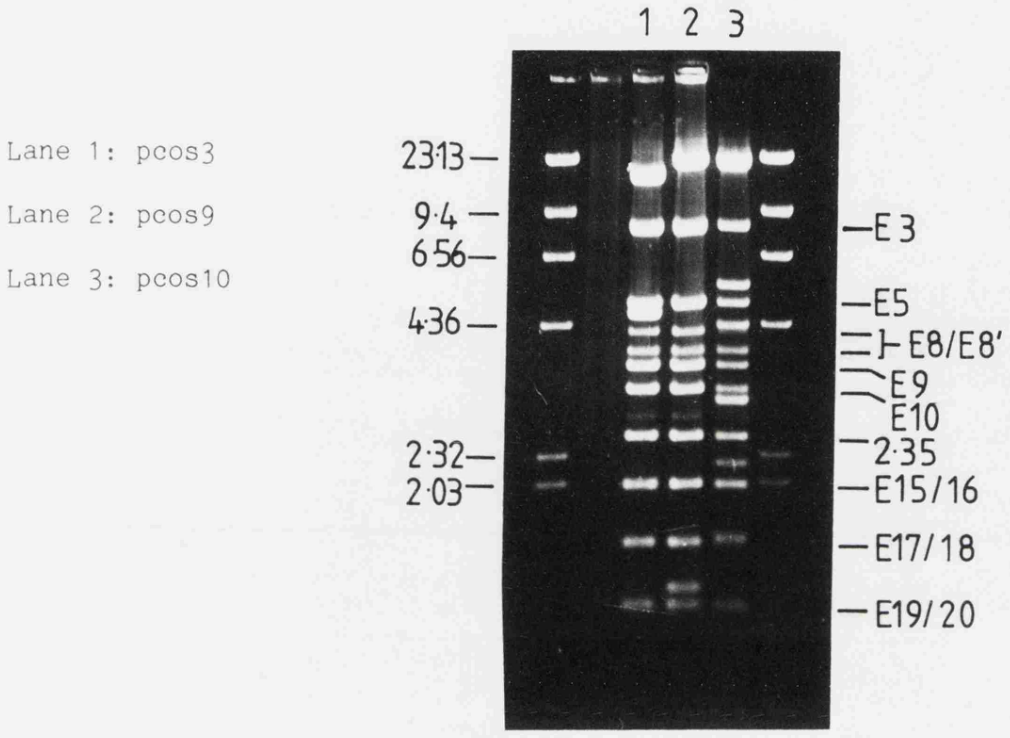
7.1 Isolation of a bank of cosmid clones.

ColIbdrd-1 DNA, partially digested with Sau3A, was ligated with Cos4 vector DNA (Figure 7.1.1) that had been cleaved with BamH1, as described in section 2.7. After packaging, 100 µl of phage suspension was used to infect 200 µl of BW86 plating cells for 15 to 20 minutes at room temperature. Once infection had occurred, the cells were added to nutrient broth and their newly acquired plasmid DNA allowed to express for 1 hour at 30°C, before selecting for ampicillin^R transfectants. Recombinant plasmids that contained the region of ColIb DNA of interest were identified by in situ colony hybridization using fragments S5 and E8 of ColIb as probes. These two fragments came from either side of the region shown by transposon insertions to be required for thin pilus synthesis. The recombinants were screened for the presence of these fragments independently. Several colonies of each type ($E8^+$ $S5^-$, $E8^+$ $S5^+$, $E8^-$ $S5^+$) were isolated and screened for production of thin pili by sensitivity to Iα phage, dnaG supression at 40°C and surface exclusion.

In conjunction with their phenotypic characterization, the recombinant plasmids were analysed by EcoRI digestion (see Figure 7.1.2). Comparison with the restriction pattern of ColIb indicated which region of the parental plasmid had been cloned and also provided a means of checking that the ColIb DNA in each case represented a contiguous fragment and not several random fragments ligated together.

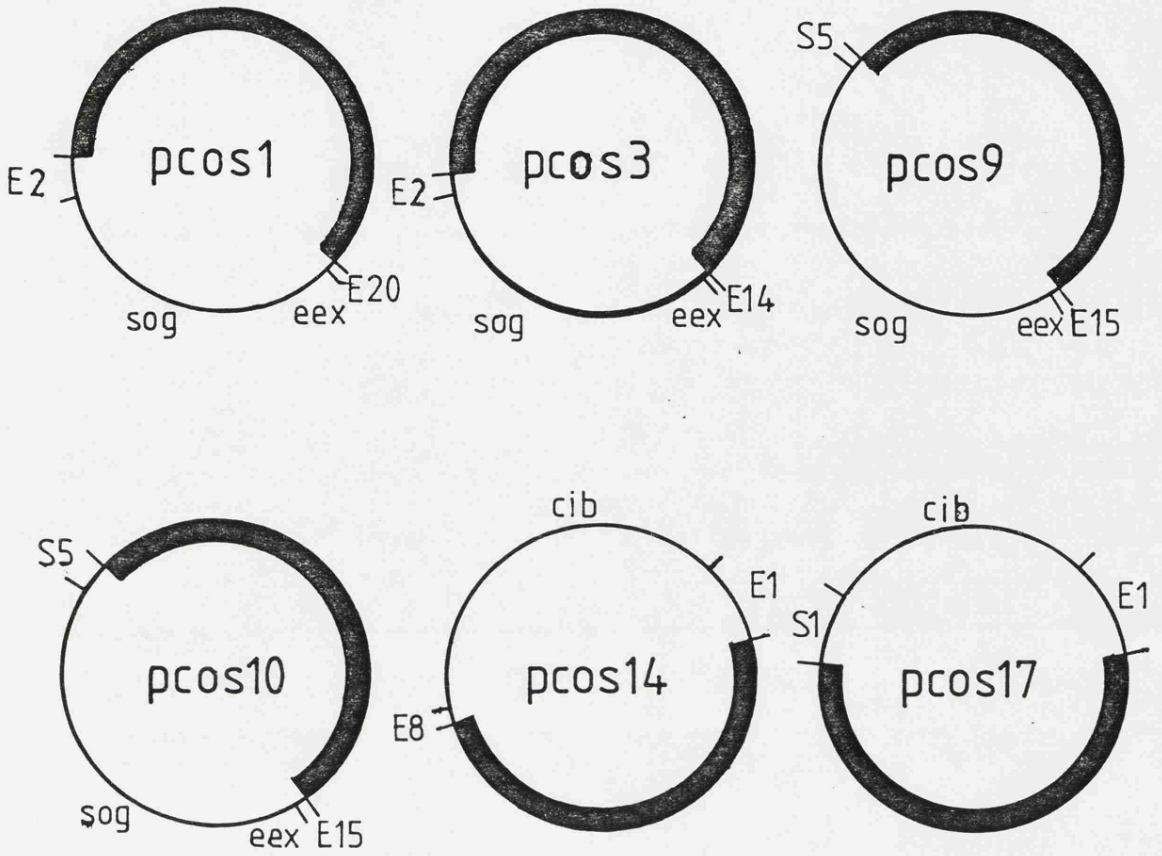
Six classes of cosmid clone were identified, as exemplified in Table 7.1.1 and the region covered by these plasmids is indicated in Figure 7.1.3. The pattern of gene expression by each of these classes corresponded closely to that expected by the region of DNA which came from ColIb. For instance, production of colicin Ib was only found in those strains carrying a segment including E12 (pcos14 and pcos17).

FIGURE 7.1.2 : EcoRI restriction of cosmid clones of ColIbdrd-1 DNA.



Those fragments that directly correspond to ColIb EcoRI fragments are indicated. Also marked is the 2.35 kb 'E13' band which results from an insertion event in E21 (see Section 7.2). The large band at the top of each track corresponds to the fraction of the E1 fragment of ColIb DNA contained in each plasmid.

FIGURE 7.1.3 : Region of ColIb DNA included in cosmid-clones.



The cosmid-clones shown exemplify the six classes of recombinant plasmids isolated by the screening technique used (see text). The diagrams are drawn in the same orientaion as the standard ColIb map. The region contained in each clone is marked by the thinner part of the circle and any relevant genes included in this DNA are also shown. The restriction fragment of ColIb in which lies the region covered by the clone is indicated at each end of the ColIb DNA. The ColIb DNA which has been deleted is indicated by the thicker part of each circle.

Similarly only cosmid-clones which contained E9 determined exclusion of IncI plasmids (pcos1, pcos3, pcos9 and pcos10). Of some 50 recombinant plasmids tested, only a strain containing pcos10 was found to express thin pili, and a more detailed examination of this clone is described in Section 7.3.

7.2 Origin of the 2.35 kb band.

When the EcoRI restriction pattern of the plasmids was studied, they were all found to contain contiguous regions of ColIb, except for the presence of a 2.35 kb band in each case, which corresponded in size to the E13 band of ColIb. Two possibilities existed to explain this finding. Firstly, E13 may have been incorrectly positioned on the EcoRI map of ColIb and in fact this fragment was located on the other side of the plasmid in the region covered by all the recombinants. If the fragment had been incorrectly mapped, the only place it could be positioned was in the E10 - E8 region, since this had not been mapped in detail (the location of E8 had only been determined by the fact that it was not included in the DNA cloned in the plasmid pCRS1a and yet was known to lie within S1). Secondly, the presence of this fragment might somehow be essential for maintenance of plasmids containing that part of ColIb which had been selected (see the kil - kor system of IncP plasmids, Figurski et al., 1985).

In an attempt to resolve these two possibilities, the position of E13 relative to the fragments E8 and E10 was determined by Southern hybridization analysis of the plasmids pCIE8 and pCIE10, using fragment E13 purified from an EcoRI restriction digest of ColIb as a probe. When this was done, the hybridization pattern of the ColIb E13 band was found to be as predicted from the map of Uemura and Mizobuchi (see Figure 1.4). However when the 2.35 kb band from pcos10 was used as a probe to the same

FIGURE 7.2.1 : Southern-blot analysis of the origin of the 2.35 kb band.

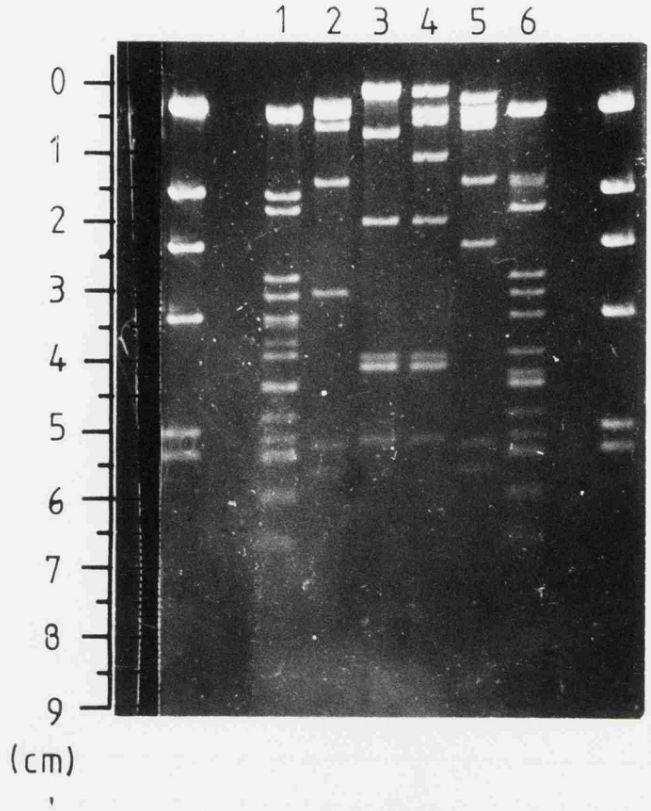
A : Restriction digests of plasmids pCIE8 and pCIE10.

Lane	DNA	Enzyme
1	pCIE10	EcoRI
2	pCIE10	SalI
3	pCIE10	HindIII
4	pCIE8	EcoRI
5	pCIE8	SalI
6	pCIE8	HindIII

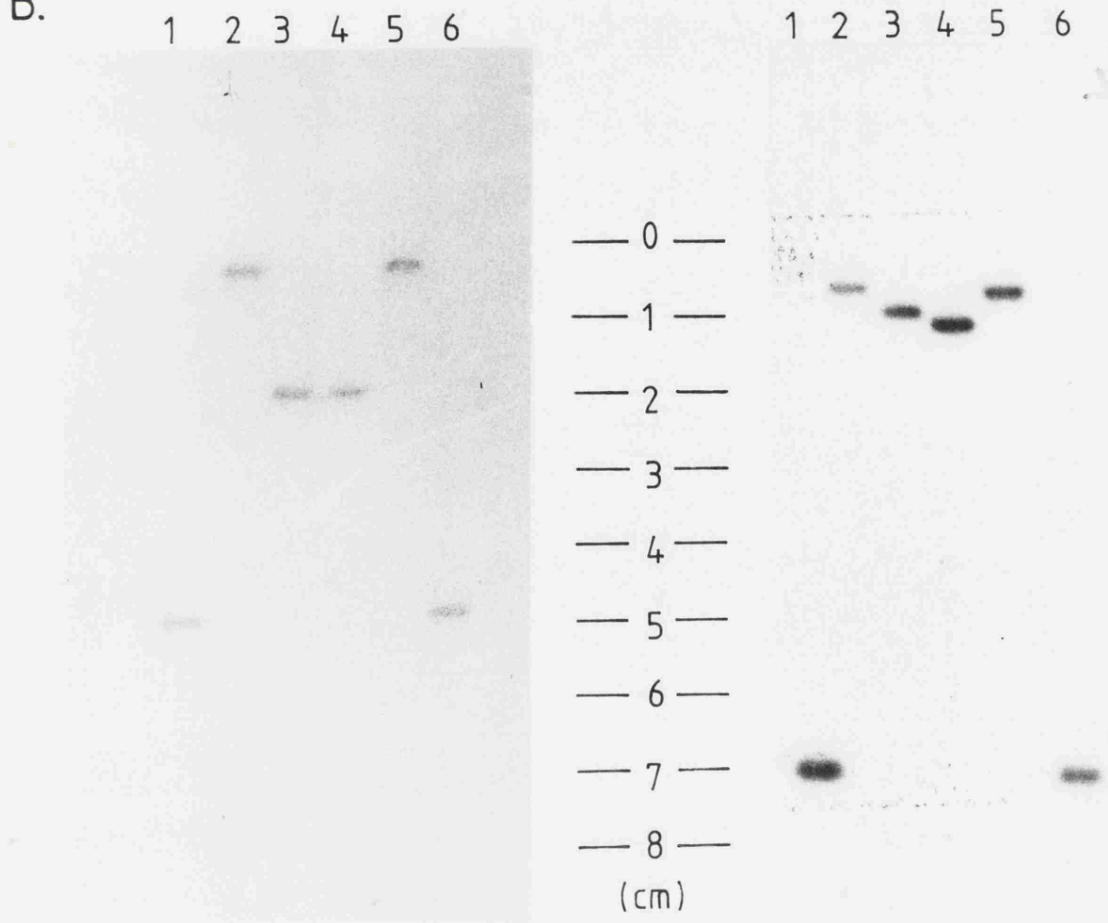
The scale to the left shows the size of the gel in cm.

B : Autoradiograms of hybridization experiments to the gel shown in A, after transfer of DNA to a nylon membrane. On the left the E13 fragment of ColI was used as a probe. On the right the 2.35 kb fragment prepared from pcos10 was used as the probe DNA. The difference in hybridization pattern shows that these are not the same fragment and that the 2.35 kb band hybridizes to fragments E21, S1a and H1b in each case (S1a is the larger half of the S1 fragment cleaved at E8 or E10 and H1b is the smaller half of H1 cleaved at E8 or E10; see Figure 1.4).

A.



B.



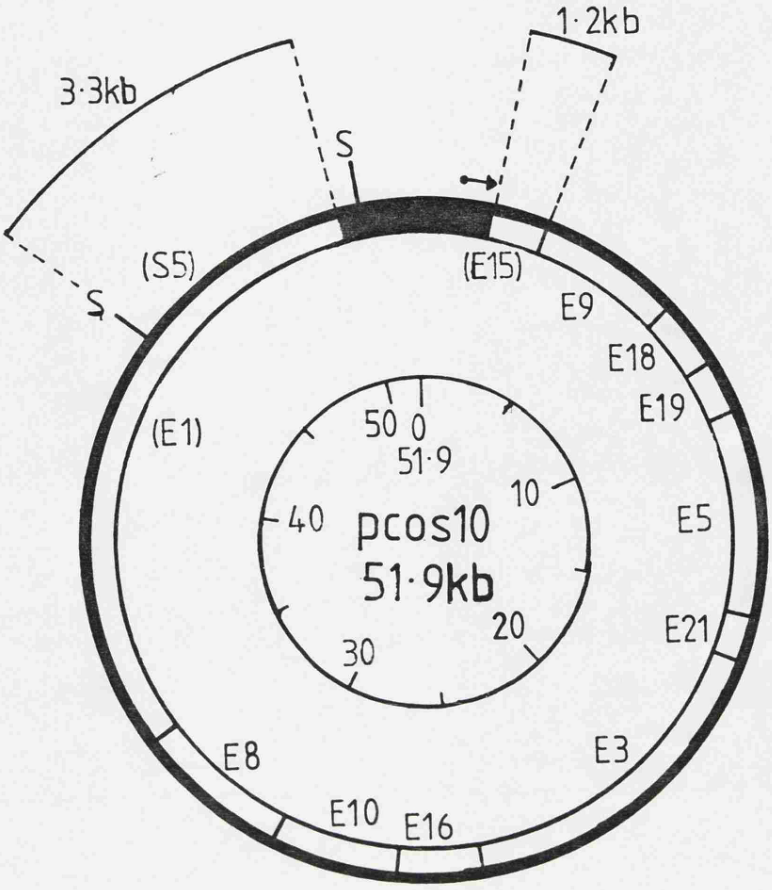
filters, this was found to hybridize to those bands which contained E21 (Figure 7.2.1). Thus it seems that the E13 fragment of ColIb was correctly located and that the 2.35 kb band is the result of an insertion event in E21 of the parent DNA used to prepare the cosmid bank. Such insertion events have been observed before in ColIb, for instance the S5 band is often found to have gained approximately 1 kb of DNA in some strains, presumably due to the acquisition of an insertion element (see Figure 4.2.2, Lane 8; the SalI fragment 5 has three forms). The E.coli chromosome contains several insertion sequences (IS) which may be the source of the extra 1.4 Kb of DNA in the cosmid-clones. In particular insertion of IS2 (1.37 kb), IS4 (1.43 kb) or IS5 (1.4 kb) (Iida et al., 1983) would produce the observed effect. The fact that pcos10 can still express both thin pili and sog primase normally, suggests that an insertion in E21 does not have any affect on the tra genes and agrees with the complementation data of Merryweather et al., 1986b), which suggests that there is a gap in the transfer genes after the sog gene.

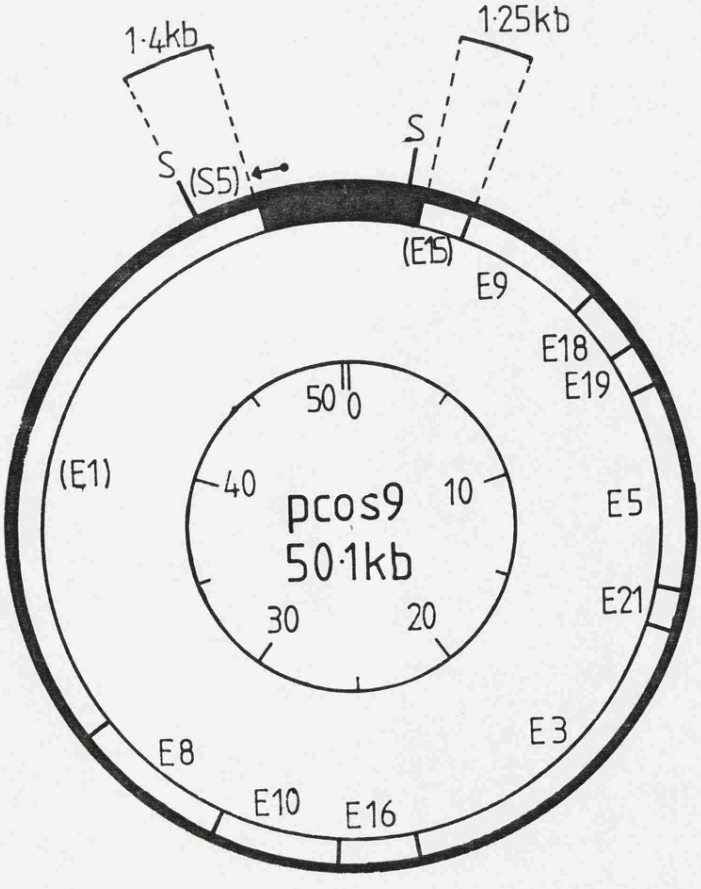
7.3 Analysis of pcos9 and pcos10.

The plasmid pcos10 was the only one from 50 recombinant plasmids tested that could express the genes required for synthesis of thin pili. When the bank of cosmid clones was prepared, the insertion in E18 had not yet been isolated and the S5 region was thought to contain the positive regulator required for transfer gene expression. Therefore it was surprising that the screening procedure employed did not identify more cosmid-clones specifying thin pili (Pil^+). However, in the light of the discovery of the second control element in E18, it is apparent that both of these regions are needed to allow expression of the tra genes. The fragments E18 and S5 lie approximately 40 kb apart, which is near that critical size of insert required to allow packaging of the

FIGURE 7.3.1 : Restriction maps of pcos9 and pcos10.

The restriction fragments marked on the circle are for EcoRI. The numbers within the circle denote the corresponding ColIb EcoRI fragment number. Numbers in brackets represent incomplete fragments. The solid line represents the vector DNA and the small arrow indicates the direction of transcription of the interrupted Tc^R gene. The vector DNA is also orientated by the single SalI site (S). The extent of ColIb fragments S5 and E15 contained within each of the cosmid-clones is shown outside the circle.


1·2kb
3·3kb
S
S
(S5)
(E15)
E9
E18
E19
(E1)
50 0
51·9
10
40
pcos10
51·9kb
E5
20
E21
30
E8
E3
E10
E16


1·25kb
1·4kb
S
S
(S5)
(E15)
E9
E18
E19
50 0
(E1)
40
10
pcos9
50·1kb
E5
E21
30
20
E3
E8
E10
E16

recombinant DNA in to phage heads. Therefore only a small proportion of the clones constructed would contain both of these regions and explains why only one Pil$^+$ plasmid was isolated.

Restriction Analysis.

To confirm that this rationale for the low number of Pil$^+$ cosmids was correct, the exact extent of ColIb*drd-1* DNA contained in pcos10, and the similar plasmid pcos9, was determined by performing double digests with the enzymes *Eco*RI and *Sal*I. This combination of enzymes separates most of the vector DNA from the ColIb DNA leaving only 0.25 kb attached to one end of the inserted DNA and 0.35 kb attached to the other (see Figure 7.1.1). Since the region of ColIb covered by these two plasmids only contains three *Sal*I sites (coordinates 40.0, 42.0 and 79.0), the *Eco*RI restriction pattern was not unduly altered and therefore it was fairly straight forward to identify the new bands generated which corresponded to the ColIb DNA attached to the vector. The results of this analysis are summarised in Figure 7.3.1.

In the region of the clones containing DNA that originated from S5 of ColIb, pcos10 was found to contain 3.3 kb of S5 DNA whilst pcos9 contained only 1.4 kb of this fragment. At the other end, both plasmids contained ColIb DNA that extended beyond the E18 fragment into E15. Therefore the major difference between these two plasmids, which presumably is the basis of the lack of expression of thin pilus genes by pcos9, seems to correlate with the amount of parental S5 DNA in the clone, again supporting the model that the S5 region is required to allow the expression of the genes required for thin pilus synthesis.

If these plasmids differ by the presence of a positive regulator however, an anomaly arises regarding the expression of the *sog* gene. Both plasmids were able to suppress the temperature-sensitive phenotype of BW86 at 40^{o}C and therefore express primase activity. No simple

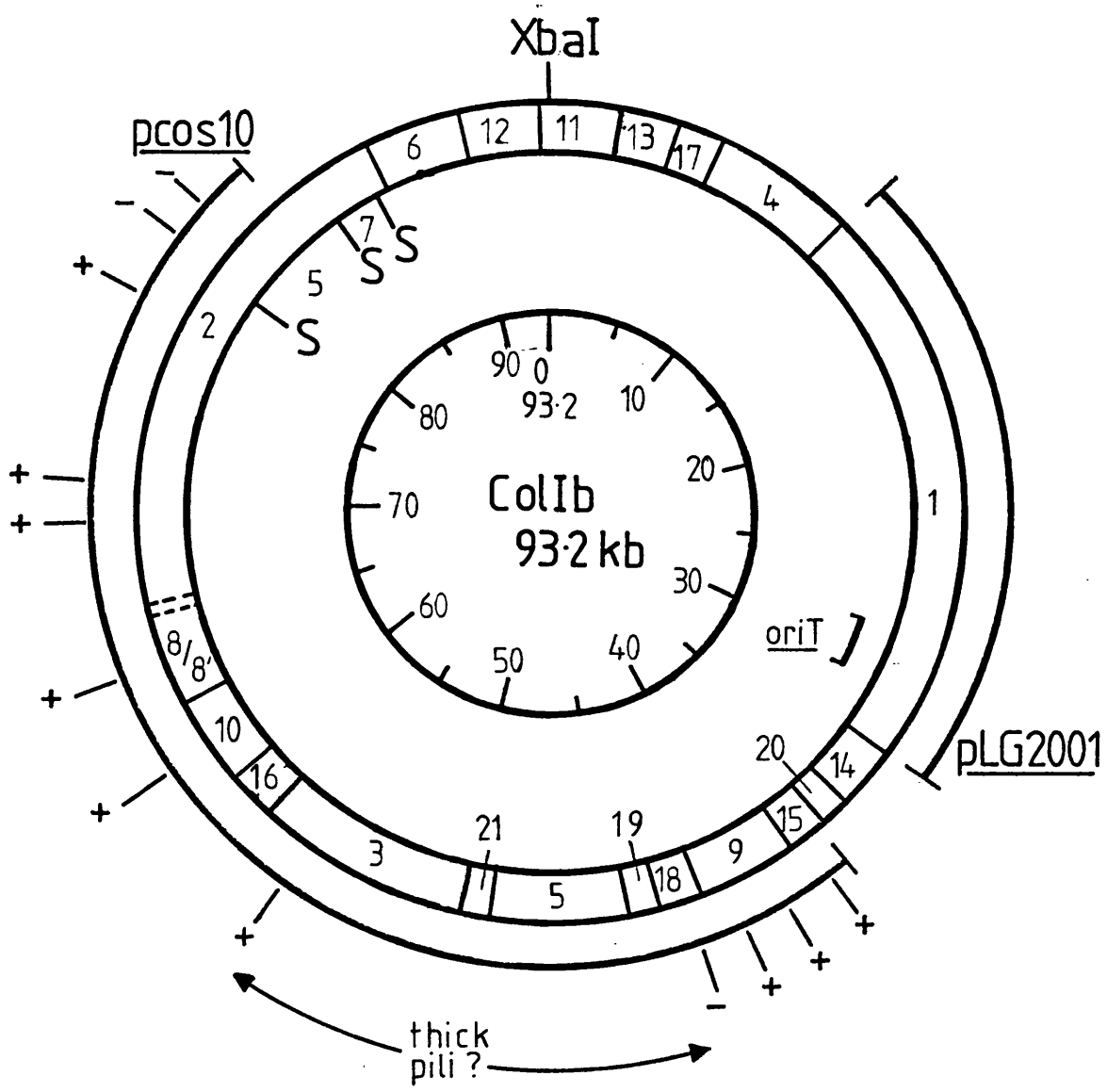
explanation exists for this disparity, but the reason may lie in the fact that mutations in the two regulator regions seem to affect the expression of different tra genes to different extents (Chapters 5 and 6). It is possible that enough of the S5 region remains in pcos9 to produce a fusion product (the Tc^R gene promoter in the vector DNA is orientated so that a fusion product could be produced) which could allow expression of the sog gene in conjunction with the trans-acting factor encoded by E18. However to explain the thin pilus-expression data, this proposed fusion product would not be capable of allowing expression of the thin pilus-genes.

Further analysis of pcos10.

Once it had been established that this plasmid expressed the thin pilus-genes, it was of interest to know whether it also expressed a complete conjugation system, although it obviously lacked the oriT site, which had been mapped beyond the end of the region of ColIb DNA covered by this cosmid-clone. Both electron microscopy studies and the ELISA test indicated that pcos10 also expressed the genes for thick pilus synthesis (pcos9 scored negatively in both tests). Taken with the fact that none of the insertions in E2, E8 and E10 abolished thick pilus biosynthesis, this delimits the region required for thick pilus expression to that between the Tn5 insertions in E3 and E15 (see Figure 7.3.2).

In a mobilization experiment however, pcos10 could not mobilize an ColIb oriT recombinant plasmid (pLG2001). The Tra^+ control plasmid, pCR9, gave a mobilization frequency of 0.29, whilst pcos10 did not promote the transfer the oriT-recombinant plasmid. Therefore some essential part of the ColIb conjugation system must be absent from pcos10, which is also not supplied by pLG2001 (the region of ColIb covered by this plasmid is indicated in Figure 7.3.2.).

FIGURE 7.3.2 : The effect of transposon insertions on the expression of thick pili.



The numbers within the circle represent EcoRI restriction fragments of ColIb. Also shown is the single XbaI site and the locations of fragments S5 and S7. Outside the circle, the extent of ColIb contained within pcos10 is shown and also the region covered in the plasmid pLG2001. The sites of transposon insertions are indicated by the lines on the outer edge of the diagram, and indicates what effect each insertion site has on the expression of thick pili (+ = normal expression, - = reduced or abolished thick pilus expression). A possible location for the coding region for thick pilus genes is indicated by the double-ended arrow.

When pcos10 was introduced in to strains carrying ColIb derivatives, it was found to be completely compatible and therefore the region of S5 contained in this plasmid must lack a complete Inc determinant. This again agrees with the finding that the Inc and Rep regions of ColIb are carried on the right half of S5, as drawn in Figure 3.3.1.

A final test was performed on pcos10 to see if the conjugation genes were subject to normal regulation. pcos10 was introduced by transformation into a strain which contained wild type ColIb-P9. The resultant strains were tested for both ampicillin-resistance and colicin production, to ensure that both plasmids were present, and for sensitivity to Iα phage. Four isolates were tested and none was found to produce thin pili. DNA was prepared from these strains and used to transform BW85. Ampicillin-resistant, colicin-negative transformants were again tested for sensitivity to Iα and all were found to express thin pili, indicating that the lack of expression by strains which also carried the ColIb*drd*$^+$ plasmid was not due to some defect in the pcos10 DNA. Rather the expression of *tra* genes from this plasmid is sensitive to normal repression.

7.4 Complementation of transposon insertion mutants of ColIb by cosmid-clones.

As demonstrated in the Section 7.3, recombinant plasmids which do not carry the incompatibility determinant in S5 can stably be maintained in the same cell as ColIb plasmids. Therefore they can be used to complement the transposon insertion mutants of ColIb. The cosmid-clones were introduced by transformation in to a *recA* strain which already carried the ColIb-derivative plasmid. These strains were used to as donors in a one hour liquid mating with BW97 to measure the rescue of transfer deficiency of the ColIb plasmid. Donors were grown in to

TABLE 7.4.1 : COMPLEMENTATION ANALYSIS.

Plasmids in donor strain	Km^R transconjugants per ml.	Rescue[a]	Efficiency[b]
pCR7	2.0×10^3		7.4×10^{-6}
pCR7,pcos3	3.0×10^4	1.5×10^1	1.1×10^{-4}
pCR7,pcos10	2.3×10^6	1.1×10^3	8.5×10^{-3}
pCR15	3.1×10^3		1.2×10^{-5}
pCR15,pcos3	9.6×10^2	0.31	3.6×10^{-6}
pCR15,pcos10	3.2×10^7	1.1×10^4	0.12
pLG264	3.1×10^5		1.2×10^{-3}
pLG264,pcos3	1.6×10^8	5.2×10^2	0.59
pLG264,pCRS1a	6.9×10^7	2.2×10^2	0.26
pCIE18	0		$<3.7 \times 10^{-9}$
pCIE18,pcos3	3.6×10^6	3.6×10^6	1.3×10^{-2}
pCIE18,pcos10	4.6×10^6	4.6×10^6	1.7×10^{-2}
pCC20	3.1×10^1		1.2×10^{-7}
pCC20,pcos3	2.5×10^1	0.81	9.3×10^{-8}
pCC20,pcos25	1.0×10^1	0.32	3.7×10^{-8}
pCC20,pcos10	6.8×10^5	2.2×10^4	2.5×10^{-3}

a : Rescue is given as the increase in frequency of transfer of the ColIb plasmid relative to the normal mutant transfer frequency of this plasmid.

b : Transfer efficiency was determined against a standard transfer frequency of 2.7×10^8 for pCR9.

exponential phase in the presence of ampicillin because it was found that, under rapid growth conditions, approximately 25% of donor cells lost the cosmid-clone. When the donor strains reached an A_{600} of 0.35, the ampicillin was removed by resuspending the cells in an equal volume of fresh nutrient broth (pre-warmed to 37°C) before mixing with the recipient cells. Transconjugant colonies were selected on L-agar plates containing nalidixic acid and kanamycin, both at a final concentration of 50 $\mu g.ml^{-1}$. The results of the complementation tests are summarised in Table 7.4.1, showing both the efficiency of transfer and the level of rescue attained in each case.

Complementation of pCR7 and pCR15.

The end point of the ColIb DNA contained in pcos3 extends approximately 3.5 kb into the E2 fragment finishing 0.5 - 0.7 kb beyond the site of the Tn1723 insertion in pCR7. When these two plasmids were present in the same cell, transfer of pCR7 was rescued 15 fold. When pcos10, which contains the all of E2 up to the beginning of the overlap with S5, was used to complement pCR7, the rescue was 1,000 fold. Two arrangements of the transfer genes affected by the insertion in pCR7 may exist. Firstly, the direction of transcription of the conjugation genes on pcos3 from the region of ColIb DNA that includes the site of the insertion in pCR7, is from right to left and they are transcribed from pcos3 either from the native promoter or from a vector promoter. The higher level of complementation by pcos10 indicates that the mutation in pCR7 can be complemented, therefore if transcription is from right to left the genes affected by the transposon insertion should be supplied by pcos3. However, the level of complementation is not as high as that achieved by pcos10 and therefore a second arrangement seems more likely. This is that transcription of the affected transfer genes of pCR7 is from left to right and, although pcos3 provides some of the downstream genes

affected by the insertion, the region of cloned ColIb DNA does not contain the whole transcriptional unit.

Where possible, the complementation tests were carried out in a recA background to reduce the possibility of recombination between the homologous DNA in the two plasmids. In one case however, the test plasmid (pCR15) could not be established in the recA strain, BW103. Therefore the mating was performed from BW85. To confirm that the transconjugants from this mating did not contain a high proportion of Tra^+ recombinant plasmids, their transfer efficiency was determined in a second mating. When this was done, all the transferred plasmid were found to have the characteristic mating frequency of pCR15, indicating that the complementation was not the result of an in vivo recombination event.

The frequency of transfer of pCR7, when complemented by pcos3, was still 10 fold greater than the frequency of transfer of pCR15. If transcription of the putative operon affected by the insertion in pCR7 is from left to right as suggested, then it would seem that the insertions in pCR7 and pCR15 do not affect the same transcriptional unit. The frequency of transfer of pCR15 was not rescued at all by pcos3 as expected since the ColIb fragment cloned in this plasmid does not extend as far as the insertion in pCR15. Complementation by pcos10 brought the efficiency of transfer of pCR15 up to near normal levels. The fact that the pCR7 transfer frequency was still 100 fold defective in the presence of pcos10 supports the model, proposed in Chapter 5, that potential pilus synthesis sites are blocked by the mutant transfer products expressed from pCR7.

Complementation of pLG264.

The Tn5 insertion mutation in pLG264 only caused a transfer deficiency of λ 87-fold and this was complemented to near normal levels by

either pcos3 or pCRS1a. This indicates that the affected *sog* polypeptides can be supplied *in trans* in the donor cell but does not provide any additional information about the arrangement of the conjugation genes in this region.

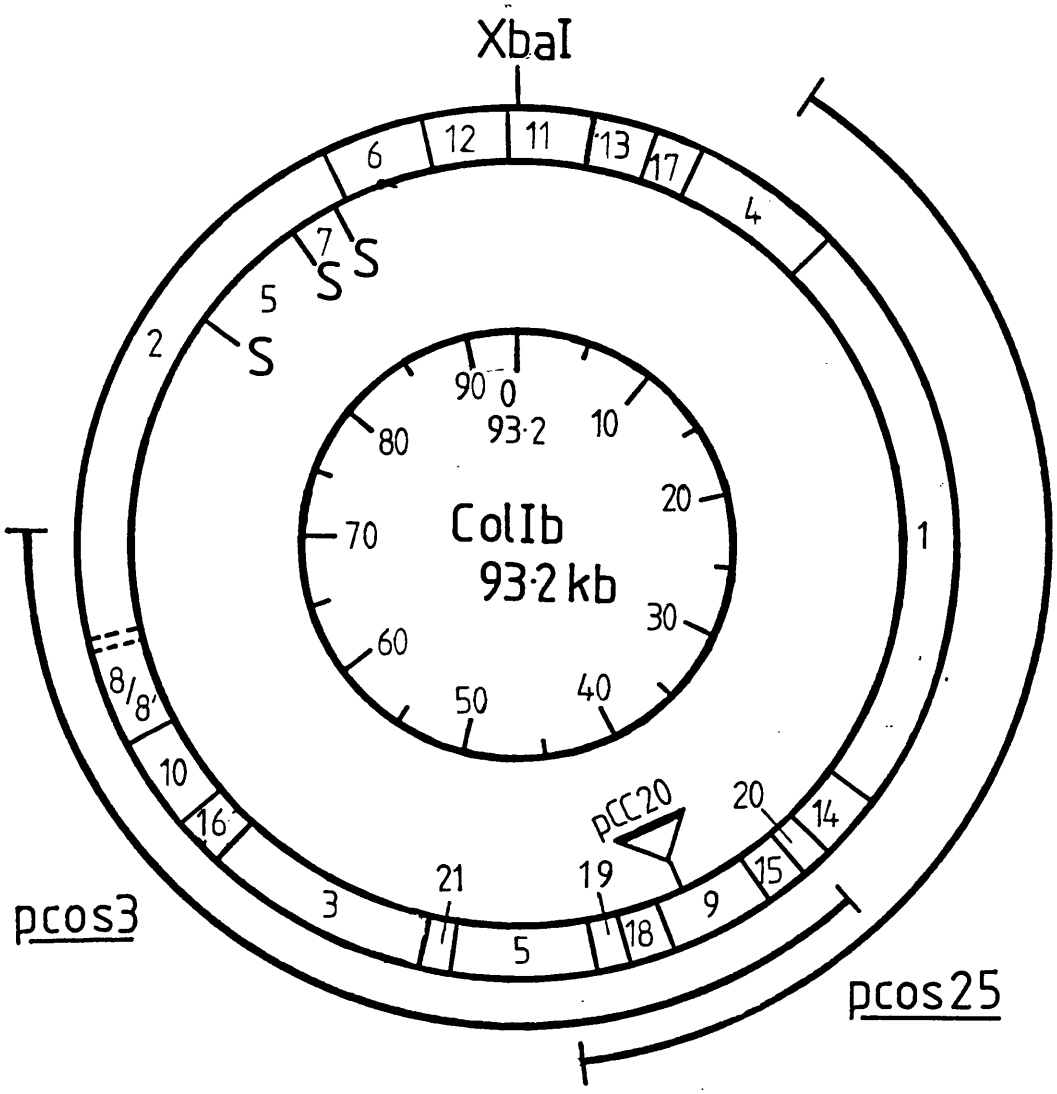
Complementation of pCIE18.

The Tn*5* insertion in pCIE18 is thought to affect a positive control factor of the ColIb conjugation genes. The fact that this mutant was complemented to within 75 - 60 fold of normal levels of transfer by either pcos3 or pcos10 suggests that *trans*-acting gene product(s) are affected rather than the disruption of a *cis*-acting site.

Complementation of pCC20.

It was proposed in Chapter 5 that the Tn*5* insertion in pCC20 does not directly affect any of the transfer functions but exerts its effect by disrupting the normal structure of the cell envelope. In agreement with this model pCC20 was not complemented either by pcos3 or pcos25, both of which contain DNA that extensively overlaps the region affected by the Tn*5* insertion (pcos3 covers all of the region of ColIb up to the *sog* gene and pcos25 [E8$^-$, S5$^-$] extends well beyond the *oriT* site; see Figure 7.4.1). However the effect of the insertion in pCC20 could only be complemented at a low level by pcos10. All the genes in the region of the insertion in pCC20 which are provided by pcos10, must also be suplied by either pcos3 or pcos25. This suggests that it is the large numbers of synthesised pili by pcos10 that overcomes the surface effects of the insertion mutation, rather than pcos10 supplying missing gene product(s). In agreement with this model, the colony morphology of BW103(pCC20,pcos10) was very large and 'fluffy' (flat and irregular) in comparison with the normally smooth colonies of BW103(pCC20), indicating that a change in surface structure had occurred. It is interesting to note that the

FIGURE 7.4.1 : Complementation of pCC20.



The numbers within the circle represent EcoRI restriction fragments of ColIb. Also shown is the single XbaI site and the locations of fragments S5 and S7. On the inside of the circle, the site of the insertion of Tn5 in pCC20 is represented. The extent of ColIb DNA carried in the two cosmids that could not complement pCC20 (pcos3 and pcos25) is indicated outside the map of ColIb. For a similar representation of pcos10, see Figure 7.3.2.

insertions which cause this affect are all immediately upstream of the eex gene. It may be speculated that the insertion events have produced a change in the level of expression of exclusion gene products and that this causes the alteration in cell-surface structure (the R144 19 Kd polypeptide has been identified as an inner membrane protein).

7.5 Summary.

Although complementation tests between pairs of transposon mutants has not been possible, complementation analysis by what are effectively deletion mutants of ColIb, has provided some insight in to the nature of the effect of the various transposon mutants tested. Unfortunately, due to the complexity of the regulation of the transfer genes, the cosmid analysis yielded less information than was hoped. However these data, along with the results from the previous chapters, can now be used to form a preliminary model of the ColIb-P9 conjugation system, which is presented in Chapter 8.

CHAPTER EIGHT : Summary and Discussion.

One of the most striking features of the ColIb conjugation system that becomes apparent is its duality. Initially it was noted that plasmids of the I complex expressed two types of conjugative pilus (Bradley, 1984). When insertion mutants were constructed that affected synthesis of the thin I_1 pilus, it was found that in many cases thick I_1 pilus expression was not affected (Chapter 5). These results provided two insights into the organisation of the transfer genes. Firstly, a region between coordinates 67.0 and 82.0 was identified which is required for the synthesis of the thin pilus. Secondly, it became apparent that the two types of pilus possessed separate assembly mechanisms. This was a somewhat unexpected result since, by analogy with F, a considerable number of tra gene products are expected to be involved in the synthesis of a sex pilus and expression of two complete sets of such genes would seem to place an unnecessarily heavy metabolic load on the host cell. Further to this finding, study of the effect of transposon insertions on the expression of the sog gene indicated that two positive regulators of the expression of the conjugation genes may exist.

Initially, studies of the plasmid pCRS1a showed that the sog gene was expressed from a promoter which was only active in the presence of a trans-acting factor provided by ColIb plasmids (Section 3.2). Later it was found that mutant plasmids with transposon insertions in either restriction fragments S5 or E18 failed to be able to supply this factor (Section 5.4). This result correlated with the observation that these transposon insertions abolished or greatly reduced sog gene expression (Section 5.2), effects which could not be the result of polarity (the insertions in S5 were known to be upstream of the sog gene promoter and that in E18 was downstream of the gene). Could it be then that these two

dual systems are related? From the findings of Dennison and Baumberg (1975) and Bradley et al (1980) it had been noted that the short rigid type of conjugative pilus do not mediate matings in liquid culture with high frequency. It may be speculated that the $IncI_1$ conjugation system has acquired the second type of pilus, the thin flexible pilus, to enhance its conjugative proficiency, and that the two-part positive regulator, a seemingly unnecessarily complex system, is a vestige of the fusion of the two systems.

Such a model may also explain the observed differences in the level of effect the individual parts of the regulator have on the expression of different transfer genes. For instance, mutations in S5 only reduce, not abolish, the expression of the thick-pilus genes but have an absolute effect on thin-pilus and nuclease gene expression. Similarly, the insertion in E18 abolishes the expression of the sog gene and of both thick and thin pili but still seems to allow the expression of the nuclease to a low level (Section 6.1). To investigate this phenomenon further it would be of interest to measure levels of mRNA produced from these regulation mutants. By using probes to the various regions containing the affected genes, it should be possible to directly quantify the level of expression of individual parts of the transfer system as directed by the different positive-regulator mutants.

Perhaps the grouping of the genes affected by each part of the positive regulator is more than fortuitous. The insertion in E8, which inactivated the nuclease gene, also seemed to prevent the rearrangement of the inversion which occurs at one end of this fragment. As was suggested at the end of Section 6.1, the location of an invertible segment adjacent to a region required for thin pilus expression, raises the possibility that this represents a biological switch that (by analogy with other such DNA inversions) somehow modifies the structure of the thin pilus. Assuming the nuc gene has some role in the recombination

event, then, if the thin pilus genes along with the S5 positive regulator were acquired as a block, it would be expected that the absence of this part of the regulator would have a more severe effect on the expression of these transfer genes than would the absence of the second part of the regulatory system. If this model is correct, then it must be assumed that the sog gene has evolved as part of a conjugation system of the type that expresses short rigid pili. In this respect it must be noted that the other well studied plasmid primase, pri, is encoded by the IncP plasmid RP4, which too encoded short rigid pili. Perhaps then the I group plasmids represent the fusion of two ancestral forms of conjugation system, one of which is typified by F and the second by RP4.

ColIb also seems to have another intermediate feature of these two types of conjugation system. The broad host range of transfer of the IncP conjugation system is well documented, whilst the host range of F group plasmids is relatively limited (Guiney, 1982; Datta and Hedges, 1972 and see Barth, 1979). The $IncI_1$ plasmids were also thought to have a narrow transfer range, but it has been shown recently that these plasmids infact have a broad transfer range but cannot be stably maintained following transfer (Boulnois et al., 1985). Perhaps then, the thick pilus genes are part of a broad transfer conjugation system, which have been acquired by an F like plasmid that still retains the narrow host range plasmid origin of replication. Possibly this could explain the association of the control of the thick pilus genes with that of the sog gene by the E18 region, since a role in broad host range transfer has been proposed for pri in facilitating the establishment of RP4 DNA in its numerous types of host cell (Lanka and Barth, 1981). The ColIb oriV is, in agreement with this model, located adjacent to the proposed thin pilus region (Section 3.3).

Whatever the scenario of its evolution, the organisation of the ColIb transfer genes is evidently complex. The fact that only one of the

transposon mutants identified was truly Tra^{o} (i.e. transfer was completely abolished) and that this was due to the mutation of a positive regulator, may reflect this complexity. Even though the two pilus assembly systems seem to be separate, they may be able to complement one another, so that many insertions would be phenotypically silent. This may account for observations such as that the insertion in fragment E20 consistently caused only a 10 fold transfer deficiency (Section 5.1), where perhaps the complementing function is not 100% efficient.

With the exception of the effect of the insertion in E20, transposon mutagenesis of the region of the plasmid that contains E20 indicated that no large blocks of transfer genes were present in this region, although the site of the unmapped insertion in pCR5 was thought to be in or near this part of the plasmid. However, the inability of pcos10 to mobilize a large ColIb-oriT recombinant plasmid which included all of the E1 fragment, suggests that some transfer functions must either extend into, or be contained within, the region surrounding E20 (see Figure 7.3.2). Their exact nature or location remains obscure but it would be of interest to see whether pcos10 could mobilize pcos25 (see Figure 7.4.1). Before this could be tested, a second resistance marker would have to be inserted into the ampicillin-resistance gene of pcos25 to allow both cosmid-clones to be stably maintained in a donor cell.

The location of the oriT site next to this region fits the pattern established from the organisation of the conjugation systems of IncF, IncN and IncP plasmids, where the origin of transfer lies at one end of the Tra region (see Figures 1.2 and 1.3). If this does represent the end of the transfer region of ColIb, and two insertions beyond this point were both Tra^{+}, then the conjugation genes of ColIb apparently account for approximately half of the plasmid, covering some 55 kb. Of course, not all of this DNA would be expected to encode tra genes, and infact one gap seems to exist between the end of the thin-pilus genes in E8 and the

sog gene (E16/E3). Also there is no direct evidence that any transfer genes lie between coordinates 42.0 and 52.0, between sog and E18, although the drd$^+$ plasmid pCIE5 was originally transfer defective when first isolated (Section 5.1). The strongest evidence perhaps that some transfer genes are contained in this region, comes from the expression of both thick and thin pili by pcos10 (Section 7.3). As shown on Figure 7.3.2 this finding, taken with the transposon insertion analysis, suggests that the thick-pilus genes are encoded between the sog gene and the fragment E18. However, the suggestion that some intra-plasmid complementation may be occurring, creates the possibility that the thick-pilus genes may be included within the region which is proposed to encode the thin-pilus genes, although the model of acquisition of one of the sets of pilus genes from another conjugation system argues against this. To resolve this debate, the best approach would now be to create deletion mutants of pcos10 in the proposed thin-pilus region, to determine if it is possible to abolish the expression of thin pili without affecting the synthesis of the thick pilus.

Finally, as described in Section 1.4, a possible correlation has been suggested between the location of plasmid-encoded gene for the single-stranded DNA binding protein (ssb) and the region of DNA which is transferred first during conjugation (Golub and Low, 1986a). The ColIb ssb gene has been shown to lie near coordinate 20.0 on the standard ColIb map (C. Howland, unpublished; see Figure 1.4). Thus, if such a correlation exists, then the direction of conjugative transfer of the ColIb plasmid DNA would be the same as that of F, so that the rest of the plasmid DNA is transferred before the conjugation genes. However, to date the evidence for such a correlation remains circumstantial and the direction of transfer of the plasmid during conjugation remains obscure.

All the data presented in this thesis may now be included in to a model of the organisation of the conjugation genes of ColIb, as shown in

Figure 8.1. Besides the results discussed so far in this chapter, also included is the evidence from the complementation studies in Chapter 7, that at least two transcriptional units are included in the thin-pilus region and that the direction of transcription of that which is interrupted by the insertion in pCR7, is from left to right, towards S5. Also shown is the direction of transcription of the sog gene, the location and proposed direction of transcription of the eex determinant and the location of the nuc gene.

One final observation that can be made from the study of pcos10 is that although the nature of the drd mutation of ColIbdrd-1 is unknown, the fact that pcos10 (which was prepared form ColIbdrd-1 DNA) was sensitive to repression by the wild type ColIb plasmid indicates that it must be a mutation in a repressor of the expression of the conjugation genes rather than a mutation causing repressor-insensitive expression of the positive regulators. This provides good evidence that the expression of the ColIb tra genes is under the same type of regulation is that of the IncF conjugation genes (i.e. negative regulation of a positively required factor).

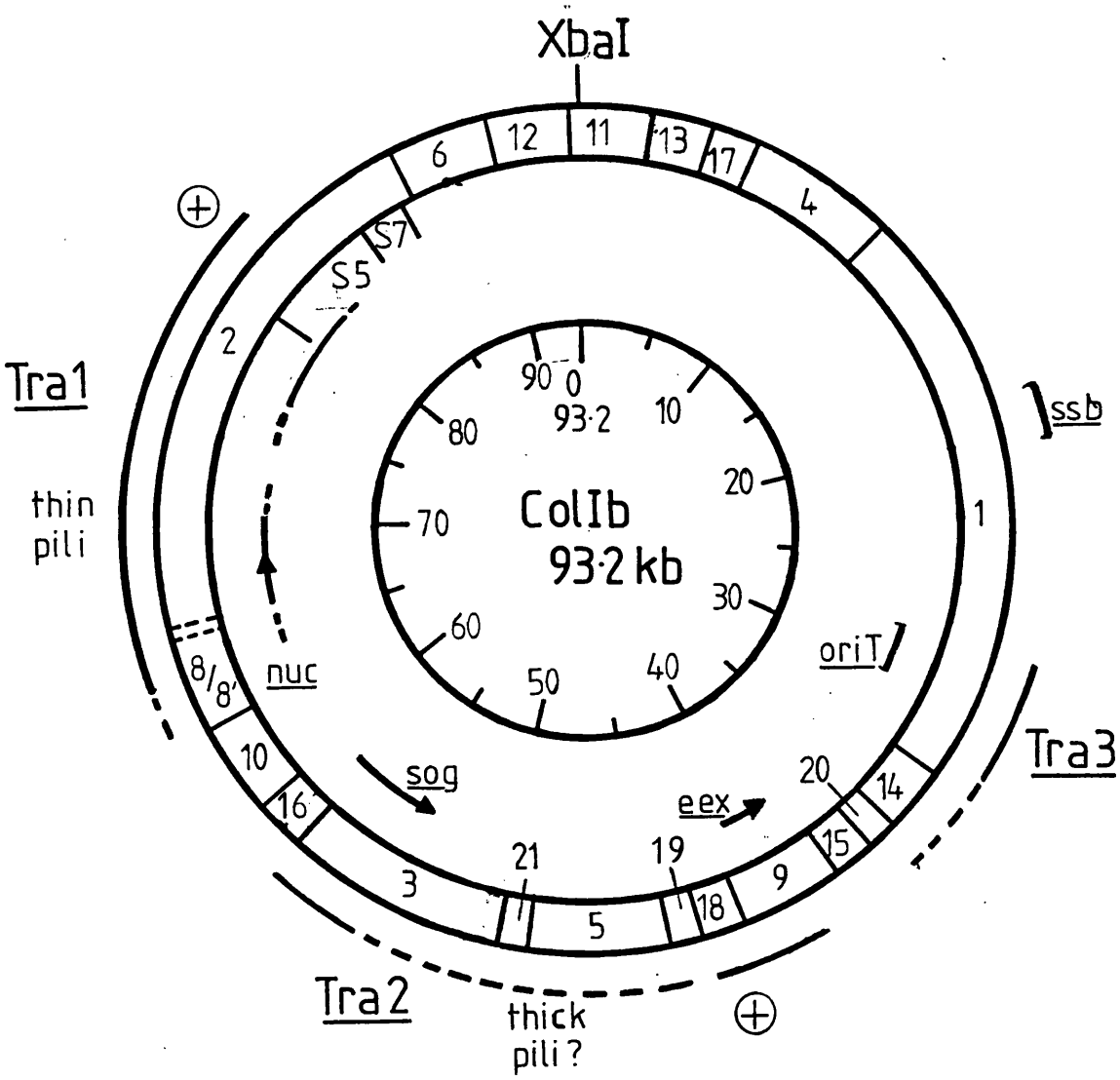
In general terms the ColIb conjugation system still displays all the essential criteria of a conjugation system deduced from the model of F described in Chapter 1 (i.e. the elaboration of sex pili, the specification of a special mechanism for the transfer of a single strand of DNA between donor and recipient cells and the expression of an exclusion system). However at the molecular level, the ColIb system is apparently far more complex, perhaps reflecting a greater versatility than that of the F conjugation system.

Bacterial plasmids from a wide range of genera are currently being studied. One of the most significant features so far identified is the prevalence of conjugation as a plasmid phenotype. This perhaps underlies the importance of conjugation as a mechanism of genetic exchange,

FIGURE 1.8 : A model of the organisation of the conjugation genes of ColIb-P9.

An EcoRI restriction map of ColIb showing the relative position of known conjugation genes. Numbers within the circle represent EcoRI fragments and also shown is the relative position of fragments S5 and S7 and the single XbaI site.

Gene symbols used are sog; plasmid-encoded primase gene, nuc; plasmid-encoded nuclease gene, eex; surface exclusion gene, oriT; origin of transfer, ssb; single-stranded DNA binding protein. Arrows are used to indicate the direction of transcription and the regions thought to contain transfer genes are designated as Tra regions and are indicated outside the circle. Also shown is the location of the two parts of the proposed positive-regulatory system, indicated by ⊕.


XbaI
ColIb
93·2 kb
93·2
0
10
20
30
40
50
60
70
80
90
Tra1
thin
pili
Tra2
thick
pili ?
Tra3
ssb
oriT
nuc
sog
eex
1
2
3
4
5
6
7
S5
S7
8/8'
9
10
11
12
13
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dramatically demonstrated by the development and spread of antibiotic resistances in pathogenic bacteria. However, it must be appreciated that one likely reason for the ubiquity of conjugative plasmids is that they represent a very successful form of evolution. During conjugative transfer, plasmid genes are necessarily replicated whilst the chromosomal genes are not. Also, once established in a cell, plasmid-encoded genes can confer either protection against antimicrobial agents or a selective growth advantage on its host, so that the host cell may proliferate at the expense of others. Whatever else, the conjugative plasmid represents a highly evolved, efficient parasite of the bacterium.

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ABSTRACT

A GENETIC ANALYSIS OF THE TRANSFER GENES OF THE IncI$_1$ PLASMID ColIb-P9.

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Plasmid ColIb-P9 is a 93.2 kb self-transmissible plasmid, belonging to the I_1 incompatibility group. Whilst much data had been gained concerning the molecular biology of conjugation mediated by this plasmid, a lack of information exsisted concerning the genetic organisation of the transfer genes.

A physical map of the plasmid was constructed by detailed restriction analysis of DNA fragments sub-cloned from ColIb-P9. These fragments were also used to locate the positions of the transfer gene *sog* and the origin of transfer. Transposons Tn*5* and Tn*1723* were used to construct insertion mutants at defined points in ColIb-P9 and the effect of these on the expression of various transfer-related functions was studied. Using this technique, the probable location of the genes encoding the thick and thin sex pili were identified and also the site of the plasmid-encoded nuclease gene. The exact location of the entry exclusion gene was also determined.

Complementation studies using the sub-cloned fragments of ColIb-P9 and a set of cosmid-clones generated from ColIb*drd-1* indicated that a positive regulator of the expression of the transfer genes exsisted and that this was composed of two genetically distinct elements. Studies involving wild type ColIb-P9 (*drd*$^+$) indicated that this positive regulatory system is subject to negative control in cells containing the *drd*$^+$ plasmid.

The information gained from these studies was combined into a model of the organisation of the transfer genes of ColIb-P9. This defines at least three separate Tra regions, covering some 50 kb of the plasmid, with the origin of transfer located at one end of the transfer region.