Tn916 insertion mutagenesis in *Escherichia coli* and *Haemophilus influenzae* type b following conjugative transfer

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Transposon Tn916 was shown to be capable of direct conjugative transfer in broth and membrane matings between strains of *Escherichia coli* K12 and between *E. coli* K12 and *Haemophilus influenzae* type b. Only Tn916 was transferred, but Tn916 donor ability was not itself inheritable by the recipients and seemed to be associated with the presence of Tn916 on a non-conjugative pBR322-derived vector in the original donor strain. Transfer of Tn916 by conjugation was found to be an efficient method for producing insertion mutations in the chromosome of recipient cells. Although such insertions were unstable when the cells were grown under non-selective conditions, it was possible to show that over 40% of the isolated Tn916 insertions in the chromosome of *E. coli* K12 were in gene(s) concerned with histidine biosynthesis, implying that there is a partial hot-spot for Tn916 insertion on the *E. coli* K12 chromosome. When a strain of *H. influenzae* type b was used as a recipient, out of approximately 1500 transconjugants tested, two mutants were isolated with insertions in genes controlling the expression of iron-regulated transferrin-binding proteins. These mutants constitutively produced major 76 kDa and minor 90 kDa proteins which bound transferrin, even when grown under iron-sufficient conditions. Tn916 insertion mutagenesis, following transfer by conjugation, is a convenient method for isolating mutations in genes concerned with iron acquisition by this important human pathogen.

Introduction

Tn916 is a 16.4 kb transposon encoding tetracycline resistance that was originally identified on the chromosome of *Enterococcus faecalis* strain DS16 (Franke & Clewell, 1981). It has been demonstrated that Tn916 is capable of conjugative transfer, in the absence of any detectable plasmid DNA, between Gram-positive genera (Clewell & Gawron-Burke, 1986), with insertions occurring at different locations on the recipient chromosome (Franke & Clewell, 1981; Gawron-Burke & Clewell, 1982). Similar conjugative transposons located on the chromosome have been found in many other Gram-positive genera (Clewell, 1990). All seem to carry a *tetM* resistance determinant (Burdett *et al.*, 1982) and share strong homology with Tn916 (Clewell & Gawron-Burke, 1986).

Previous studies have demonstrated that Tn916 can be introduced by transformation into *Escherichia coli* K12 following *in vitro* ligation with pGL101, a pBR322-derived vector (Gawron-Burke & Clewell, 1984). The

recombinant plasmid, designated pAM120, was unstable in *E. coli* K12 under non-selective conditions, but prolonged growth in the absence of tetracycline was required before Tn916 insertions in the *E. coli* chromosome could be detected (Gawron-Burke & Clewell, 1984). In contrast, Tn916 introduced into *Streptococcus sanguis* on a shuttle vector not only excises from the plasmid, but also inserts readily into the chromosome, thereby serving as a convenient insertion mutagen (Gawron-Burke & Clewell, 1984). Similarly, in *Haemophilus influenzae*, *H. parainfluenzae* and *Neisseria meningitidis*, it has been shown that Tn916 will insert at random locations in the recipient chromosome following the introduction of pAM120 by transformation (Kauc & Goodgal, 1989; Kathariou *et al.*, 1990). The use of pAM120 as a transposon delivery system is particularly efficient because the vector plasmid is unable to replicate in these genera.

Conjugative transfer of Tn916 was originally demonstrated between Gram-positive bacteria. More recently, conjugative transfer of Tn916 has also been reported to occur between Gram-positive and Gram-negative bacteria (Bertram *et al.*, 1991). In view of the behaviour of

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Fig. 1. Restriction map of pAM120 showing the position of the *tetM* gene. Thick line, pGL101 DNA; thin line, Tn916 DNA; E, *EcoRI*; Hc, *HincII*; Hd, *HindIII*; Kp, *KpnI*.

Table 1. Characteristics of bacteria and plasmids used in this study

Bacteria	
<i>E. coli</i> K12	
HB101	F ⁻ <i>recA pro lacY ara gal xyl mtl rpsL supE hsdS</i>
J53.2	F ⁻ <i>pro met rpoB</i>
KT1035	F ⁻ <i>trp lacZ thi rpsL</i>
KT1300	HB101(pAM120)
<i>H. influenzae</i> type b	
JKP1	Strain Eagan; invasive clinical isolate
JKP2	Streptomycin-resistant mutant of JKP1
JKP3	Rifampicin-resistant mutant of JKP1
JKP13	Tn916 insertion mutant of JKP3
JKP14	Tn916 insertion mutant of JKP3
Plasmids	
pGL101	Amp ^r
pAM120	pGL101::Tn916 (Amp ^r , Tet ^r)

Tn916 in certain Gram-negative bacteria following transformation experiments, the primary objective of this work was to assess the potential for Tn916 insertion mutagenesis in *Escherichia coli* and *H. influenzae* following direct conjugative transfer. In the present paper we: (a) report the conditions required to achieve successful transfer; (b) describe the fate of Tn916 in the recipient cells; and (c) use Tn916 to isolate insertion mutants in the genes controlling the expression of transferrin-binding proteins in *H. influenzae* type b.

Methods

Bacteria and plasmids. The strains of bacteria and plasmids used in this study are listed in Table 1. Strains of *E. coli* were maintained on airtight nutrient agar slopes at room temperature. Strains of *H. influenzae* type b were maintained at -70°C in Brain Heart Infusion broth (Oxoid) supplemented with NAD (Sigma; $2\mu\text{g ml}^{-1}$), protoporphyrin IX (PPIX; Sigma; 500 ng ml^{-1}) and glycerol (20%, v/v).

Growth conditions. For experiments in liquid media, strains of *E. coli* were grown with aeration in nutrient broth (NB) at 37°C . Strains of *H. influenzae* were grown with aeration in Brain Heart Infusion broth supplemented with NAD and PPIX as above (BHINP). For some experiments, growth under iron-restricted conditions was achieved by the addition of the iron-chelator ethylenediamine-di-*o*-(hydroxyphenylacetic acid) (EDDA; Sigma) to BHINP as described previously (Morton & Williams, 1989). Nutrient agar (NA) and 'chocolate' blood agar (CBA) were used to subculture *E. coli* and *H. influenzae* strains, respectively. Antibiotics (Sigma) used for selection purposes were

incorporated into liquid and solid media at the various concentrations described in Results.

Conjugation experiments. Donor strains containing pAM120 were grown initially on agar plates containing tetracycline HCl ($4\mu\text{g ml}^{-1}$), and were then subcultured into 10 ml NB or BHINP, both also containing tetracycline HCl ($4\mu\text{g ml}^{-1}$). Following overnight growth at 37°C , 1 ml was inoculated into 10 ml fresh antibiotic-containing broth and incubated with gentle shaking at 37°C for 3 h. A culture of the recipient strain was prepared similarly in antibiotic-free broth. For broth matings, donor cultures were centrifuged ($5000g$ for 15 min) and the cells resuspended in the same volume of antibiotic-free NB or BHINP immediately before mixing 1:1 with the recipient culture. Incubation was under static conditions at 37°C . Membrane filter matings were as described by Towner & Vivian (1976), but with overnight incubation of the filter at 37°C on the surface of a CBA plate. Selection of Tn916 transconjugants was on either NA (for *E. coli*) or CBA (for *H. influenzae*), both containing appropriate concentrations of antibiotics (see Results). DNAase I (Sigma; beef pancreas) was dissolved in 0.02 M-MgSO₄ and added to certain mating mixtures at a final concentration of $400\mu\text{g ml}^{-1}$. All plates were incubated at 37°C .

Isolation of DNA. Plasmid DNA was isolated as described by Silhavy *et al.* (1984), and visualized in 0.7% agarose gels. Total cellular DNA from *H. influenzae* was isolated by a method based on that of Moxon *et al.* (1984). Briefly, cells grown overnight at 37°C on a shaking incubator in 20 ml BHINP were harvested by centrifugation and resuspended in 3 ml lysis buffer (0.1 M-NaCl, 10 mM-Tris/HCl, pH 8.0, 10 mM-EDTA). Aliquots of 500 μl were transferred to microfuge tubes and 50 μl 10% (w/v) SDS was added to each tube. The tubes were inverted rapidly three or four times to lyse the cells, and then heated at 60°C for 10 min. The tubes were cooled and 27.5 μl proteinase K solution (Sigma; 20 mg ml^{-1} in H₂O) were added to each tube and the contents mixed by inversion. Following incubation at 37°C for 1 h, the contents of each tube were extracted twice with 577.5 μl Tris-buffered phenol (saving the top aqueous layer), followed by one extraction with 577.5 μl water-saturated butanol (saving the bottom aqueous layer). The DNA was then precipitated by the addition of twice its volume of absolute ethanol, and stored at -20°C until required.

Preparation of labelled probes. Two DNA probes were used (Fig. 1): (i) the 1.5 kb *HindIII/KpnI* fragment of pAM120 which is internal to the *tetM* gene of Tn916 (Senghas *et al.*, 1988); (ii) the 2.4 kb *EcoRI* fragment of pAM120 comprising pGL101 vector DNA and excluding Tn916 (Gawron-Burke & Clewell, 1984). Probe DNA was prepared, purified and labelled with biotin-14-dATP (Gibco BRL) as described by Carter *et al.* (1987).

Hybridization experiments. Dot-blots of total cellular DNA extracted from *H. influenzae* were prepared as described by Williams *et al.* (1990). Cultures of *E. coli* were filtered on to nitrocellulose membranes and treated as described by Carter *et al.* (1989). Plasmid DNA was transferred from agarose gels to nitrocellulose membranes by electroblotting (Towner *et al.*, 1988). Membranes were air-dried and then baked at 80°C for 2 h to bind DNA to the membrane. Before hybridization, dot-blots were rehydrated and treated with proteinase K

as described by Carter *et al.* (1987). Prehybridization and hybridization with one of the labelled probes was also done as described by Carter *et al.* (1987). Detection of a positive hybridization result was by means of a BlueGENE kit (Gibco BRL) using the conditions and protocols recommended by the manufacturer.

Screening of transconjugants for insertion mutations. *E. coli* J53.2 transconjugants were first patched on to NA plates containing tetracycline ($10 \mu\text{g ml}^{-1}$) and rifampicin ($100 \mu\text{g ml}^{-1}$), incubated at 37°C overnight, and replica-plated on to plates of *E. coli* minimal medium (Vogel & Bonner, 1956) containing glucose (0.5%), proline ($50 \mu\text{g ml}^{-1}$) and methionine ($50 \mu\text{g ml}^{-1}$). Auxotrophic mutants unable to grow on the supplemented minimal medium were recovered from the master plates and screened for additional auxotrophic requirements as described by Holliday (1956). Lack of a defined minimal medium meant that it was not possible to screen transconjugants of *H. influenzae* for the acquisition of auxotrophic requirements. However, as part of a parallel study, transconjugants of *H. influenzae* JKP3 were screened for Tn916 insertions in genes controlling expression of outer-membrane transferrin-binding proteins (Morton & Williams, 1990; Holland *et al.* 1991). Colony lifts on nitrocellulose membranes (Sambrook *et al.*, 1989) were screened for the ability of transconjugants, grown on the iron-sufficient selection medium, to bind human transferrin in the solid-phase dot enzyme assay employing horse-radish-peroxidase-(HRP)-labelled transferrin, as described by Morton & Williams (1990). The original selection plates were reincubated at 37°C to allow regrowth of colonies. Those colonies which corresponded with a positive transferrin-binding reaction on the nitrocellulose membranes were subcultured on fresh plates of selection medium. Transferrin-binding ability was confirmed by spotting cells resuspended in 10 mM-Tris/HCl buffer (pH 7.4) on to nitrocellulose, and then repeating the dot enzyme assay.

Affinity isolation and electrophoresis of transferrin-binding proteins. Transferrin-binding proteins were isolated from *H. influenzae* essentially as described by Schryvers (1989) and modified by Holland *et al.* (1991). Briefly, bacterial cell envelopes prepared by sonication of whole cells were first incubated with biotinylated human transferrin. The biotinylated transferrin/transferrin-binding protein complexes were then solubilized from the membrane with 50 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-NaCl, 2% (w/v) sodium *N*-lauroyl sarcosinate (Sarkosyl; Sigma) and 10 mM-EDTA. The complexes were isolated with streptavidine immobilized on agarose beads (Sigma); these were collected, resuspended in SDS-PAGE sample buffer (without 2-mercaptoethanol) and incubated for 30 min at 37°C . After centrifugation to remove the beads, samples were applied to 10% (w/v) SDS-polyacrylamide gels (approximately $2 \mu\text{g}$ protein per lane). Following electrophoresis, proteins were electroblotted on to nitrocellulose and probed with HRP-labelled transferrin as described above for the dot enzyme assay.

Stability of insertion mutations. Mutant strains carrying a Tn916 insertion were grown at 37°C overnight in 10 ml of appropriate antibiotic-free broth. Cultures of auxotrophic mutants derived from *E. coli* J53.2 were centrifuged and then resuspended in the original volume of $\frac{1}{4}$ -Ringer's solution. Portions (0.1 ml) of this suspension were spread on to plates of *E. coli* minimal medium containing glucose, proline and methionine to detect revertants to the original J53.2 phenotype. Viable counts were determined by spreading appropriate dilutions on to plates of NA. Appropriate dilutions of *H. influenzae* JKP3 derivatives carrying Tn916 insertions in genes controlling expression of transferrin-binding proteins were spread on to plates of antibiotic-free CBA. Single colonies were patched on to fresh plates of antibiotic-free CBA and then tested for their ability to bind transferrin by spotting cells resuspended in 10 mM-Tris/HCl buffer (pH 7.4) on to nitrocellulose and repeating the dot enzyme assay described above.

Fig. 2. Example of data obtained with a single donor culture of *E. coli* KT1300 in broth conjugation experiments at 37°C . The same culture of KT1300 was mated with *E. coli* J53.2 (circles) or *H. influenzae* JKP3 (squares) under static (open symbols) or vigorously shaken (filled symbols) conditions.

Results

Conjugative transfer of *pAM120*

Broth and filter mating experiments were performed between *E. coli* strains KT1300 and J53.2, and between KT1300 and *H. influenzae* JKP3. In view of the poor initial expression in *E. coli* and *H. influenzae* of the *tetM* resistance determinant encoded by Tn916 (Courvalin & Carrier, 1987; Kauc & Goodgal, 1989), it was considered probable that conjugative transfer of Tn916, if it occurred, would be detectable only with selective tetracycline concentrations marginally above those that would normally be inhibitory for the recipient. Therefore, selection for J53.2 transconjugants was made on NA plates containing rifampicin ($100 \mu\text{g ml}^{-1}$) and tetracycline ($10 \mu\text{g ml}^{-1}$), while selection for JKP3 transconjugants was on CBA plates containing rifampicin ($50 \mu\text{g ml}^{-1}$) and tetracycline ($4 \mu\text{g ml}^{-1}$).

Fig. 2 shows examples of the data obtained in broth matings performed with a single donor culture of *E. coli* KT1300. Incubation of selective plates at 37°C for 48–96 h was required for transconjugant colonies to develop. The fact that very few transconjugants were obtained when the mating mixtures were shaken vigorously was indicative that transfer of genetic material occurred by conjugation. No significant reduction in the number of transconjugants was observed when mating mixtures contained DNAase I. No colonies were obtained when a cell-free lysate of the donor strain was used instead of intact donor cells.

There was a wide variation in the number of transconjugants generated in broth mating experiments involving different cultures of the donor strain. This observation may simply reflect the varied efficiencies obtainable in broth as opposed to solid surface matings,

but may also be a consequence of the known instability of pAM120 in *E. coli* K12 (Gawron-Burke & Clewell, 1984). Although donor cells were cultured in the presence of tetracycline before mating, the mating mixtures themselves did not contain tetracycline. Control experiments demonstrated that only 3% of donor cells formed tetracycline-resistant colonies following growth for 6 h in tetracycline-free broth. The observed variations in the number of transconjugants obtained in broth matings may, therefore, result from rapid and random loss of pAM120 from donor cells before pair formation with recipient cells can occur (see also Discussion). Membrane filter matings generated more reproducible results, with approximately 10^3 transconjugants per plate in matings between strains of *E. coli* K12, and approximately 50 transconjugants per plate in matings between *E. coli* K12 and *H. influenzae* JKP3.

Fate of pAM120 in transconjugants

Tetracycline-resistant transconjugants of *E. coli* J53.2 and *H. influenzae* JKP3 were purified by restreaking on to fresh plates of the initial selection medium. These cultures were then used as donors in mating experiments with *E. coli* KT1035 and *H. influenzae* JKP2. Selection for KT1035 transconjugants was on NA plates containing streptomycin ($100 \mu\text{g ml}^{-1}$) and tetracycline ($10 \mu\text{g ml}^{-1}$), while selection for JKP2 transconjugants was on CBA plates containing streptomycin ($100 \mu\text{g ml}^{-1}$) and tetracycline ($4 \mu\text{g ml}^{-1}$). No colonies were obtained in any of these mating experiments, indicating that the donor ability associated with pAM120 was not itself inherited by the initial transconjugants.

In order to examine further the fate of pAM120 in the initial recipient strains, 153 tetracycline-resistant J53.2 transconjugants and 300 tetracycline-resistant JKP3 transconjugants were patched and regrown on their respective isolation medium and then replica-plated to NA or CBA containing ampicillin ($50 \mu\text{g ml}^{-1}$). All the transconjugants tested were ampicillin-sensitive. This suggested that the pGL101 portion of pAM120, encoding ampicillin resistance, had been lost from the transconjugants. Since the pGL101 portion contains the origin of replication of pAM120, this result also indicated that Tn916, encoding tetracycline resistance, must have excised from pAM120 and inserted into another replicon in the recipient strain.

Plasmid and DNA probe experiments

Agarose gel electrophoresis was used to examine 10 J53.2 and 10 JKP3 tetracycline-resistant transconju-

gants for the presence of plasmid DNA. No plasmid DNA was detected in the J53.2 transconjugants, but the original JKP3 recipient strain, together with all 10 transconjugants tested, contained a plasmid of approximately 29 kb. Plasmid DNA from the JKP3 transconjugants was transferred by electroblotting to nitrocellulose membranes and probed with the 1.5 kb *Hind*III/*Kpn*I fragment (internal to Tn916) of pAM120. No hybridization was detected, indicating that in each of the J53.2 and JKP3 transconjugants tested, Tn916 had probably inserted into the chromosome.

To examine this point further, dot-blots of total cellular DNA from the J53.2 and JKP3 transconjugants were probed with either the 1.5 kb *Hind*III/*Kpn*I fragment of pAM120 (internal to Tn916) or the 2.4 kb *Eco*RI fragment of pAM120 (external to Tn916). No hybridization was observed with the 2.4 kb *Eco*RI fragment, whereas all transconjugants tested hybridized with the 1.5 kb *Hind*III/*Kpn*I fragment. No hybridization of this fragment to total cellular DNA from the original J53.2 culture was detected, but some hybridization was detected with total cellular DNA from the original JKP3 culture (see Discussion).

Isolation and characterization of Tn916 insertion mutants

E. coli

A total of 153 tetracycline-resistant *E. coli* J53.2 transconjugants were screened for the acquisition of additional auxotrophic requirements. Of these, 74 failed to grow on initial screening. Further examination revealed that 66 had an additional auxotrophic requirement for histidine, while the other 8 had reverted back to the J53.2 phenotype. It therefore appeared that Tn916 inserted preferentially into gene(s) concerned with histidine biosynthesis on the chromosome of *E. coli* K12.

H. influenzae

H. influenzae type b strain JKP1 binds transferrin only after growth in iron-restricted media (Morton & Williams, 1990). Tn916 transconjugants were therefore screened for constitutive expression of transferrin binding after growth in iron-sufficient media. Colony lifts of approximately 1500 *H. influenzae* JKP3 transconjugants grown on an iron-sufficient medium were screened for their ability to bind transferrin. Of these, 6 colonies gave an apparent positive result. These colonies were purified by restreaking and were then retested using cells resuspended in Tris/HCl buffer. Only two transconjugants (designated JKP13 and JKP14) bound transferrin consistently when grown in iron-sufficient medium (Fig. 3a).

Fig. 3. (a) Dot enzyme assay showing the binding of an HRP-human transferrin conjugate to whole cells of the Tn916 insertion mutants JKP13 (lane A) and JKP14 (lane B), together with the parent strain JKP3 (lanes C and D). Bacteria were grown in iron-sufficient medium (BHINP; lanes A, B and D) or in iron-restricted medium (BHINP + 100 μ M-EDDA; lane C). (b) Western blot showing binding of an HRP-transferrin conjugate to affinity-isolated transferrin-binding proteins from the Tn916 insertion mutants JKP13 (lane A) and JKP14 (lane B), together with the parent strain JKP3 (lanes C and D). Transferrin-binding proteins were prepared from bacteria grown in iron-sufficient medium (BHINP; lanes A, B and D) or in iron-restricted medium (BHINP + 100 μ M-EDDA; lane C). Molecular masses of transferrin-binding proteins were calculated by comparison with the migration of molecular mass markers in the range 30–200 kDa (Sigma).

Previous work has shown that the interaction between transferrin and *H. influenzae* involves at least one iron-regulated outer-membrane transferrin-binding protein (Schryvers, 1989; Morton & Williams, 1990; Holland *et al.*, 1991). Therefore, the transferrin-binding proteins of JKP13 and JKP14, isolated after growth in iron-sufficient medium (Fig. 3b, lanes A and B), were compared with those isolated from the parent strain, JKP3, grown under iron-restricted (lane C) and iron-sufficient (lane D) conditions. Both mutants constitutively produced major 76 kDa and minor 90 kDa proteins which bound HRP-transferrin (i.e. even when grown under iron-sufficient conditions), while JKP3 expressed these proteins only after growth in media

containing the synthetic iron-chelator EDDA. It was concluded that Tn916 had disrupted a gene(s) concerned with control of expression of the transferrin-binding property.

Stability of insertion mutants

Ten *his* derivatives of *E. coli* J53.2, each carrying a Tn916 insertion, were investigated. In no case were revertants to the original J53.2 phenotype detected (reversion frequency of $<1.8 \times 10^{-9}$). In contrast, reversion of the *H. influenzae* mutants JKP13 and JKP14 was detected at high frequency. Of 100 colonies of JKP13 grown on antibiotic-free CBA, only 7 retained the constitutive transferrin-binding phenotype and concomitant tetracycline resistance. The remaining 93 colonies were tetracycline-sensitive, showing that Tn916 had excised from the chromosome. JKP14 was considerably more stable: of 100 colonies grown on antibiotic-free CBA, 98 retained tetracycline resistance and the constitutive transferrin-binding phenotype.

Discussion

Tn916 is a member of a closely-related family of conjugative transposons which were identified originally in Gram-positive bacteria (Clewett & Gawron-Burke, 1986). These elements are found normally on the bacterial chromosome, but are able to transpose intracellularly to resident plasmids. It has long been known that Tn916 is capable of conjugative transfer between numerous Gram-positive genera (Clewett & Gawron-Burke, 1986) and, more recently, it has been demonstrated that direct conjugative transfer can occur between Gram-positive and Gram-negative bacteria (Bertram *et al.*, 1991). The present study provides the first evidence that Tn916, when cloned on a pBR322-derived vector in *E. coli* K12, is capable of direct conjugative transfer to other Gram-negative recipient strains. It should, however, be noted that tetracycline-resistant transconjugants were detected only after prolonged incubation on selective media, possibly as a result of poor expression in recipient cells of the *tetM* resistance determinant encoded by Tn916 (Courvalin & Carlier, 1987; Kauc & Goodgal, 1989).

It was of particular interest that the initial *E. coli* K12 transconjugants received Tn916 (inserted apparently into the chromosome) but not the vector plasmid, and that the transconjugants carrying a copy of Tn916 were unable to act as new donors. This is in contrast to the situation observed following the introduction of an intact copy of pAM120 into a new *E. coli* host by transformation (Gawron-Burke & Clewett, 1984; this paper). This

difference might be a copy number effect since pAM120, carrying Tn916, is a multicopy plasmid with a pBR322-derived origin of replication (Gawron-Burke & Clewell, 1984), and presumably exists in about 20 copies per cell, while only one copy of Tn916 exists on the chromosome of each transconjugant. This could result in a transfer frequency below the limit of detection. An alternative possibility is that the vector plasmid itself has some role in initiating Tn916 conjugation in *E. coli* K12. This could also partly explain the varied mating efficiencies observed with broth cultures. It is known that pAM120 (pGL101 carrying Tn916) is unstable in *E. coli* K12 (Gawron-Burke & Clewell, 1984). If an association between pGL101 and Tn916 is necessary for Tn916 conjugation to be initiated in an *E. coli* K12 donor, the efficiencies of transfer observed would be a reflection of the proportion of donor cells in the mating mixture that carried an intact copy of pAM120.

It has been shown previously that Tn916 can be used as an insertion mutagen in several Gram-negative genera following its introduction by transformation (Kauc & Goodgal, 1989; Kathariou *et al.*, 1990). Transfer of Tn916 by conjugation is an easier and efficient method for producing insertion mutations. When *E. coli* K12 strain J53.2 was used as a recipient, it was possible to demonstrate with DNA probes that Tn916 had inserted into the chromosome. In addition, more than 40% of the isolated Tn916 insertions were found to be in gene(s) concerned with histidine biosynthesis, implying that there is a partial hot-spot for Tn916 insertion on the *E. coli* K12 chromosome. Possible hot-spots for Tn916 insertions have been suggested previously for *Clostridium acetobutylicum* (Woolley *et al.*, 1989) and *C. difficile* (Mullany *et al.*, 1991). In contrast, Kauc & Goodgal (1989) have suggested that Tn916 integrates into many different sites in the *H. influenzae* genome. When *H. influenzae* JKP3 was used as the recipient, it was not possible to demonstrate unequivocally with DNA probes that Tn916 had inserted into the chromosome. Despite the fact that the original strain of JKP3 was tetracycline-sensitive, some hybridization was detected with the internal *tetM* fragment from Tn916 that was used as a probe. The *tetM* determinant has been shown to occur naturally in a wide range of urogenital and respiratory bacteria, including *Haemophilus* (Roberts, 1990). It is, therefore, possible that a partial or unexpressed sequence of *tetM* is already present on the chromosome of JKP3.

One of the original aims of this work was to find a convenient mutagenesis method for *H. influenzae* that involved insertion of a detectable DNA sequence into particular genes of interest. High affinity iron-sequestering systems are known to contribute to bacterial virulence by facilitating the acquisition of this essential bacterial nutrient in the iron-restricted conditions en-

countered in mammalian body fluids (Hennecke, 1990; Griffiths, 1991). The lack of readily available iron in body fluids appears to constitute a major environmental signal for pathogens causing infection, controlling not only expression of iron-uptake mechanisms, but also global regulation of other virulence factors such as toxin production (Hennecke, 1990). Certain human pathogens, notably *N. meningitidis* and *H. influenzae* type b, have been shown recently to express siderophore-independent iron-sequestering systems based on a direct interaction between host iron-binding glycoproteins, such as transferrin, and bacterial surface receptors (Tsai *et al.*, 1988; Schryvers, 1989; Ala'Aladeen *et al.*, 1990; Griffiths *et al.*, 1990; Morton & Williams, 1990). *H. influenzae* JKP3 produces one major iron-regulated outer membrane protein which can be renatured on Western blots to bind human transferrin (Morton & Williams, 1990; Holland *et al.*, 1991). Mutants lacking this protein are unable to bind transferrin or utilize transferrin-bound iron (Holland *et al.*, 1991). Interestingly, whilst the transferrin-binding proteins are iron-regulated in laboratory-adapted *H. influenzae* type b strains, a survey of fresh clinical isolates has revealed that they, like the Tn916 insertion mutants JKP13 and JKP14, show constitutive expression of these proteins (unpublished observations). The transferrin-binding proteins of such isolates become iron-regulated only after up to 40 *in vitro* subcultures in iron-rich medium, a finding which suggests that the transferrin iron-uptake system is expressed *in vivo* during infection.

The Tn916 insertion mutants JKP13 and JKP14, which showed constitutive expression of the outer membrane transferrin-binding proteins, were unstable in non-selective conditions. However, it should be possible to use pulsed-field electrophoresis techniques, in conjunction with cells grown under selective conditions, to locate precisely the positions of these insertion mutations on the *H. influenzae* chromosome (Kauc *et al.*, 1989; Butler & Moxon, 1990). Conjugation is a simple and effective way of producing such mutations. Their ready availability should enable progress to be made in understanding the regulatory mechanisms involved in this potentially important virulence determinant.

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