An *in silico* evaluation of Tn916 as a tool for generalized mutagenesis in *Haemophilus influenzae* Rd

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The transposon Tn916 was evaluated as a tool for generalized mutagenesis of the genome of *Haemophilus influenzae*. This was achieved *in silico* by searching the genome sequence of *H. influenzae* Rd for the published Tn916 target site consensus sequence 5' TT/ATTT(N)₆AAAAA/TA. This search identified 16 putative target sites. In subsequent experiments, integration of Tn916 did not occur at any of these sites. Using the nucleotide sequences of these observed integration sites, a new consensus sequence, 5' TTTT(N)_xAAAAA (4≤x≤7), was derived. This sequence reflects the curve–twist–curve DNA topology which is a feature common to all Tn916 integration sites. A search of the *H. influenzae* Rd genome using the new consensus sequence identified 167 potential target sites, representing approximately 1% of the total genome. Only 80 of these sites were located within ORFs. The presence of such a limited number of target sites places severe constraints on the use of Tn916 as a tool for generalized mutagenesis of the genome of *H. influenzae*.

Keywords: *Haemophilus influenzae* Rd, Tn916 mutagenesis, genome sequence

INTRODUCTION

Haemophilus influenzae is a cause of both upper and lower respiratory tract infections (Turk, 1984). Strains expressing capsular serotype b also cause invasive disease such as meningitis. Recently, the complete genome sequence of *H. influenzae* Rd, an avirulent, non-encapsulated derivative of a serotype d strain, was determined. This information represents a powerful tool with which to study the biology of this organism (Fleischmann *et al.*, 1995). Identification of ORFs in the *H. influenzae* Rd genome has been achieved by comparison of their nucleic acid and amino acid sequences with those of known genes and proteins. Of the 1743 predicted coding sequences identified in *H. influenzae* Rd, 736 have not been assigned a function. No database matches have been found for 389 of these, whilst 347 have homology to hypothetical proteins which occur in other organisms. Thus, the function of many genes remains undetermined.

The role of key virulence determinants in the development of invasive disease, such as capsule and LPS, have been well characterized. However, invasive disease

is a complex process and there are many steps which remain to be explored. To further characterize this process, methods are required to generate a representative set of mutants from which those with increased or decreased virulence can be selected. One strategy which is traditionally considered to fulfil this criterion is transposon mutagenesis. Naturally occurring transposons have not been identified in *Haemophilus* species. However, transposon Tn916 has been successfully introduced into the genomes of both *H. influenzae* and *H. parainfluenzae* by transformation using the suicide plasmid pAM120 (Kauc & Goodgal, 1989) and by conjugation (Holland *et al.*, 1992). Tn916 is an 18 kb conjugative transposon originally identified in the chromosome of *Enterococcus faecalis* (Franke & Clewell, 1981). It is a member of a family of closely related conjugative transposons which includes Tn1545 from *Streptococcus pneumoniae* (Courvalin & Carlier, 1987). These transposons are promiscuous and are able to function in a wide range of bacteria (Clewell *et al.*, 1995). They have been used successfully to generate mutants deficient in both LPS and capsule biosynthesis in *Neisseria meningitidis* (Stephens *et al.*, 1991; Kahler *et al.*, 1996). Although Tn916 is capable of transposition

in *H. influenzae*, it does not generate the comprehensive range of mutations typical of a transposon mutagenesis experiment. When this strategy was used to identify genes involved in LPS biosynthesis, mutations in genes directly involved were not recovered, although a mutant with an altered LPS phenotype was generated (High *et al.*, 1996). This suggested that the number of sites into which Tn916 could integrate in the *H. influenzae* genome was limited, although the production of mutants with the required phenotype was possible.

Integration of Tn916 into host DNA has been reported to occur at target sequences with the consensus 5' TT/ATTTT(N)₆AAAAAA/TA, where (N)₆ represents any 6-base sequence (Lu & Churchwood, 1995). This consensus was derived by examination of 24 target sites (compiled by Scott *et al.*, 1994) used by Tn916 in *Streptococcus pyogenes*, *Listeria monocytogenes*, *Bacillus subtilis*, *Ent. faecalis* and *N. meningitidis*. This sequence has similarity with the nucleotide sequence of the right- (RH) and left-hand (LH) ends of Tn916. A characteristic of Tn916 target sequences is that they are predicted to contain static bends centred at the site of transposon insertion. This DNA topology is thought to facilitate binding of the integrase encoded by Tn916 and thereby integration of the transposon into the host genome (Lu & Churchwood, 1995). No single base in the consensus integration sequence is conserved amongst all target sequences analysed to date and individual targets can differ from this common sequence at several positions whilst retaining the ability to interact with integrase. Since transposition of Tn916 is not a random event, its use as a tool for generating mutations in the genome of *H. influenzae* and other organisms will, consequently, be limited by the number and distribution of target sequences.

METHODS

Bacterial strains, culture conditions and plasmids. *H. influenzae* Rd is an avirulent, non-encapsulated derivative of a serotype d strain (Leidy *et al.*, 1960). *Escherichia coli* strain CG120 contains the chimeric plasmid pAM120/pGL101 into which Tn916 has been cloned (Gawron-Burke & Clewell, 1984).

H. influenzae strains were cultured in brain heart infusion (BHI) broth (Oxoid) supplemented with haemin (10 µg ml⁻¹) and NAD (2 µg ml⁻¹). Solid Levinthal's BHI medium was prepared by the addition of Bacto agar (1%, w/v) and Levinthal base (Alexander, 1965). *E. coli* CG120 was grown in Luria broth supplemented with ampicillin (25 µg ml⁻¹) and tetracycline (4 µg ml⁻¹). *H. influenzae* Tn916 transformants were cultured in the presence of tetracycline (4 µg ml⁻¹).

Transformation of *H. influenzae* with Tn916. *H. influenzae* was transformed with pAM120 using the M-IV procedure (Herriot *et al.*, 1970). In each transformation 1 µg DNA was used to ensure that DNA was not limiting. Tn916 transformants were selected by growth on Levinthal's BHI medium containing tetracycline (2 µg ml⁻¹). A transformation efficiency of 3.5 × 10⁻³ was routinely achieved.

PCR amplification of Tn916-chromosomal DNA junction regions. Recognition sites for the restriction enzyme *SspI*

Fig. 1. Restriction map of Tn916 showing the relative positions of the *SspI* and *Asp718* restriction endonuclease sites used in this study. The position and direction of the primers used for PCR amplification of the LH and RH Tn916-chromosomal DNA junction regions are shown. The box indicates the location of *tetM*, the tetracycline resistance gene present in Tn916. The dashed lines either side of Tn916 represent *H. influenzae* chromosomal DNA.

occur frequently in the genome of *H. influenzae*, and 1613 and 203 bp in from the LH and RH extremities of Tn916, respectively (Fig. 1). By digesting chromosomal DNA from each mutant with *SspI*, two small restriction fragments harbouring the chromosomal DNA-Tn916 junctions were generated. The digested chromosomal DNA was then ligated under conditions that favoured intramolecular ligation. The ligated DNA was used as the template in an inverse PCR and the nucleotide sequence of the amplified product determined. The nucleotide sequences of the primers used to amplify the junction between chromosomal DNA and the LH extremity of Tn916 were 5' GAAGTATCTTCCTACAGT (Oligo A) and 5' CCTCTGCCAAGCGTCGTAGC (Oligo B). 5' TTTGATACGACGATTTATCCT (Oligo C) and 5' TGATAAAGTGTGATAAGTCCA (Oligo D) were used to amplify the junction between chromosomal DNA and the RH extremity of Tn916 (Fig. 1). Primers A and C were biotinylated.

Direct sequencing of PCR products. Biotinylated PCR products were bound to streptavidin-coated Dynabeads (Dyna). The bound PCR product was denatured with 150 mM NaOH, washed three times in sterile distilled water and then re-suspended in 14 µl distilled water. An aliquot (7 µl) of this Dynabead-bound PCR product was then used for sequencing which was performed as outlined in the USB Sequenase handbook.

RESULTS

The number and distribution of potential Tn916 target sites on the genome of *H. influenzae*

To assess whether Tn916 transposition is an effective method for generating mutations in *H. influenzae*, the number and distribution of potential Tn916 target sites on the genome of *H. influenzae* Rd was determined. The genome sequence was searched for sequences which matched the Tn916 target site consensus sequence, 5' TT/ATTTT(N)₆AAAAAA/TA, using the EGCG program FindPatterns which locates short nucleotide sequence patterns (Devereux *et al.*, 1984; <http://www.sanger.ac.uk/Software/EGCG>). Only 16 sequences which matched this consensus were identified. Since the target site consensus sequence does not represent every potential target sequence at which Tn916

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Tn916  pATGCGGATAAACTAGATTTTTATGCTATTTTNNNNNNAACAAAGTATAAATTTCTAATTATCTTTTATATTT
Int 1   AGTTGTGAAATTTGCAGTTCGTCACAAT TTTTGTATAAAA ACAGATATAATCGACGCAATATGGCAAATTTAT
Int 2   ATTTTCATCAGCGCTAACTTATTAGTA TTTTAATATAAA GTTGTGTGTTCCCTGCTTGGATATAAGTTGCATTA
Int 3   TTTTGAAGATACTTTTGTACTACTA TTTTTCACAGGAAAA ATCAAGAAAATTATCAGTCAAGTTAAGAAC
Int 4   GTTGTAAAAAGTTTGCAGTTCGTCACAAT TTTTGAATAAAAA CAGATATAATCGACGCAATATTTCATCCTGT
Int 5   GGGGTTGAGTTGTCAATTATTATTCTCT TTTTAGCATACAAAA TTGTGCTTATCTTACTTTAAATAGGGGATT
Int 6   GCCAAAAGGCGGTGCGTTTTCTGTAT TTTTAGATAAAAA AACCGCAACTTATAAAGTGCGGGTTAAATTGG
Int 7   ATTTGAAATAAGTTAAAATGATGAAAA TTTTAATAGGAAAA TAATGAAAACAGCTGAAAATTCAACCGCACTT
Int 8   GGTTTAGGTTTGTATTCTTTTATTTT TTTTAGCAAGAAAA ATATTCAAATTTTATTGGAACATCTTTTATAT
Int 9   TTTTAATGAAAGAAAATATTTTATTA TTTTCACTCTAAAA GTGAACTTTTTCTAAAGTTATTAGATATTCCTC
Int 10  TTGTCAAAAATTTGACTATTTTCAAG TTTTTTAACATAAAAA TTGTTTTATTGAAAAATGTTTAAATTAACCT
Int 11  AATTTTGTAAATTGTTGTTTAATACGA TTTTAAATTTAAAA TGGCACCGCCTACAATAAATAACCCTAGGAAA
Int 12  TCATTATTTCAAGTATCATTTGCTTAT TTTTACGATTAAAA GGAAATAGCAATGAAATGTAAGCGTTTAAATG

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Fig. 2. Nucleotide sequences of the LH–RH junction region of the circular intermediate form of Tn916 and the integration sites of Tn916 identified in this study. The region corresponding to the consensus sequence 5' TTTT(N)₆AAAA 3' (4 ≤ x ≤ 7) is separated from the main body of the sequence by a single space. NNNNNN represents the variable coupling sequence present in Tn916. The additional tract of 5 dT residues present in the LH–RH junction region of the circular intermediate form of Tn916 and in target site Int 6 is highlighted in bold.

can insert, this figure is only a conservative estimate of the number of such sites on the genome of *H. influenzae*. Nevertheless, it indicated that potential Tn916 integration sites were not widely represented.

Identification of Tn916 integration sites in *H. influenzae*

To test whether the above prediction was accurate, it was necessary to determine how effective the consensus sequence was in identifying Tn916 integration sites in *H. influenzae*. To achieve this Tn916 was introduced onto the genome of *H. influenzae* in two independent transformation experiments. Eighteen different transformants were selected arbitrarily, without regard for any alteration in phenotype and the relative location of Tn916 in each was determined by Southern hybridization. Genomic DNA from each transformant was digested with *Asp*718 which cuts once in Tn916 and *Xba*I that does not cut within the transposon. This generated two DNA fragments containing the LH and RH ends of Tn916 and the associated flanking DNA. Digested genomic DNA from each transformant was then probed with Tn916. Twelve distinct hybridization patterns were apparent in the 18 transformants analysed (data not shown), suggesting that Tn916 had integrated into only 12 different genomic locations. In all cases each mutant only contained one copy of the transposon. One hybridization pattern occurred a total of five times in both transformation experiments, suggesting that Tn916 had inserted into an integration 'hot-spot'.

Determination of the nucleotide sequence of Tn916 integration sites

The site at which Tn916 had integrated into the genome of each mutant was determined by sequencing the Tn916–chromosomal DNA junctions (Fig. 2). By comparison of each sequence to the genome sequence of *H. influenzae* Rd, the location of each target site and the identity of any ORF that had been disrupted was established (Table 1). As suggested by Southern hybridization,

only 12 different Tn916 insertion mutants had been generated. Of these, 10 were located within intergenic regions (Table 1). A total of five Tn916 insertions occurred at the target site Int 6 in two independent transformation experiments. Three of these insertions were derived from the first transformation and the remaining two from the second transformation. Two insertions at target sites Int 5 and Int 8 were observed. In the case of Int 5, both insertions were obtained from the same transformation. Given that a transformation efficiency of 3.5×10^{-3} (mg DNA)⁻¹ was routinely obtained and the cells were only incubated for 20 min prior to plating out on selective media, it is unlikely that the two Int 5 transformants are siblings.

Searching the *H. influenzae* Rd genome sequence with a modified Tn916 target site consensus sequence

None of the target sites identified in this study matched the published consensus sequence. A consensus sequence reflecting the characteristics of the integration sites identified in this study was therefore derived to obtain a more accurate estimate of the number and location of potential Tn916 target sites in the Rd genome.

Each of the Tn916 integration sites was analysed using the programs DNA+ and RasMol which calculate the phase and degree of curvature of a nucleotide sequence (DeSantis *et al.*, 1996; Sayle & Milner-White, 1995). All of the integration sites were predicted to form curved DNA structures containing a phase change, resulting in a curve–twist–curve structure (Fig. 3). The curve in the target sites is generated by the short tracts of dA and dT on either side of the central (N)₆ sequence. When these occur on different faces of the helix, the overall direction of curvature, or phase, is different on either side of the coupling sequence, introducing a twist into the helix. Int 6, at which five Tn916 insertions had occurred, was predicted to have the greatest DNA curvature due to the presence of an additional homopolymeric tract of 5 dT residues with preserved helical facing, located 6 bp 5' to

Table 1. The location of each transposon integration site on the *H. influenzae* Rd genome

Target site	Location of insertion on the Rd genome	ORF disrupted	Identity of nearest ORFs		Location of ORFs on genome
Int 1	1317346–1317410	No	HI1244	No database match	1317246–1316629
			HI1245	Malic acid enzyme	1317444–1319711
Int 2	387337–387411	Yes	HI0364	Penicillin-binding protein 5 precursor	387935–387060
Int 3	1727530–1727605	No	HI1660	Ribonucleoside diphosphate reductase	1726333–1727460
			HI1661	Dihydrolipoamide succinyltransferase	1728808–1727582
Int 4	1317347–1317422	No	HI0985	DNA processing chain	1042260–1041085
			HI0986	Isopropylmalate synthetase	1042715–1044307
Int 5	1353302–1353375	No	HI1276	Methionine tRNA synthetase	1353315–1351270
			HI1277	Putative ATPase (Mrp)	1353418–1354575
Int 6	1042650–1042710	No	HI0985	DNA processing chain	1042206–1041085
			HI0986	Isopropylmalate synthetase	1042715–1044307
Int 7	1218830–1218900	No	HI1151	Hypothetical protein	218780–1218824
			HI1152	PmbA	1218869–1220221
Int 8	1808470–1808510	No	HI733	RNase II	1810602–1807374
			HI732	AidA-1 adhesin	1808267–1807374
Int 9	1616560–1616630	No	HI546	Hypothetical protein	1616761–1617180
			HI547	DAHPSynthetase	1618360–1617275
Int 10	581150–581250	No	HI10561	Hypothetical protein	581046–579148
			HI10562	Hypothetical protein	581360–581752
Int 11	209569–209644	Yes	HI0195	Hypothetical protein	208082–211414
Int 12	892797–892893	No	HI10844	Guanidine dinucleotide biosynthesis	892784–892209
			HI10845	Hypothetical protein	892871–893134

Fig. 3. DNA secondary structure formed by (a) integration site Int 6 (angle of curvature 131°), (b) the Tn916 LH–RH junction region of the circular intermediate form of Tn916 (121°) and (c) integration site Int 2 (138°). The nucleotide sequence used to generate this data is illustrated in Fig. 2. The graphic output and the degree of DNA curvature of each target site was obtained using the program RasMol (Sayle & Milner-White, 1995).

the TTTT(T)₆AAAAA sequence. An equivalent homopolymeric tract is also present at the same position in the Tn916 LH–RH junction region (Fig. 2). The presence of this sequence would have the effect of increasing the degree of DNA curvature within this region relative to other target sites. This observation may explain why Int 6 acts as a ‘hot-spot’ for Tn916 insertion.

In 8 of the 12 Tn916 integration sites the curve–twist–curve structure coincided with the presence of the motif 5′ TTTT(T)₆AAAAA at the centre of the integration site, where (N)₆ represents the coupling sequence and is composed of any base. In the remaining sites it is formed by equivalent tracts of dA and dT separated by either 4, 5 or 7 nt. When the contribution of the polyA and polyT tracts to the observed curvature is considered, the sequence 5′ TTTT(T)_xAAAAA (4 ≤ x ≤ 7) more accurately describes the determinants of local topology associated with Tn916 integration sites. Only one integration site, Int 2, did not fit this pattern, although it was predicted to form a similar secondary structure to the other integration sites identified (Fig. 3). Comparison to the consensus sequence derived by Lu & Churchwood (1995) revealed that our modified consensus matched 50% of the 16 target sites that it had identified. When the *H. influenzae* Rd genome sequence was searched with the new consensus, 167 matches were found of which 80 were located in ORFs.

DISCUSSION

Although it is well established that the transposon Tn916 can integrate into the genome of *H. influenzae*, it has not proven to be an effective tool for generating mutations with defined phenotypes in this organism. This has led to the suggestion that the number of integration sites for Tn916 in the genome of *H. influenzae* might be limited. Experiments carried out by

Kauc & Goodgal (1989) determined the location of Tn916 integration sites on the genome of *H. influenzae* Rd by agarose gel electrophoresis of *Apa*I- and *Sma*I-digested genomic DNA. Although this strategy confirmed that Tn916 could integrate into the *H. influenzae* genome, the precise location of each integration site could not be defined. The completion of the *H. influenzae* Rd genome sequence provided the first opportunity to determine the number and relative distribution of Tn916 integration sites in this organism. Only 16 potential Tn916 target sites which fitted the Tn916 integration site consensus sequence (Lu & Churchwood, 1995) were identified. These sites were not used by Tn916 in subsequent transformation experiments, indicating that it could integrate into the Rd genome at sites which did not fit the consensus sequence. To obtain a more accurate estimate of the number of potential target sites, a new, more comprehensive consensus sequence was derived through comparison of the actual target sites used by the transposon in *H. influenzae*.

A feature of the Tn916 integration sites identified in this study is that they are predicted to form a characteristic curve-twist-curve secondary structure. A similar structure is also formed by the LH-RH junction of Tn916 which binds directly to integrase. DNA topology may therefore play an important role in mediating integration of Tn916 into host DNA. A similar curve-twist-curve structure is also an important component of the target sites used by the *Bacillus subtilis* insertion element IS231A, although in this case the structure is formed by tracts of poly-dC and poly-dG (Hallet *et al.*, 1994). The extent of DNA curvature also appears to influence the frequency with which sites are used as targets for Tn916 integration. Tn916 appears to insert preferentially into target sites such as Int 6 which have a relatively high degree of DNA curvature. The DNA curvature at the Int 6 integration site is similar to that formed by the Tn916 LH-RH junction region which is involved in binding integrase (Fig. 3). The secondary structure of Int 6 may therefore result in an optimal interaction with integrase which would explain why Tn916 integration occurs here more frequently than at other target sites. The affinity with which a target site binds integrase and hence the frequency with which it experiences Tn916 insertion may therefore be dictated in part by its secondary structure (Lu & Churchwood, 1995).

Since the Tn916 integration sites identified in *H. influenzae* have a conserved DNA topology, it was concluded that a consensus sequence reflecting this feature might identify a more comprehensive range of integration sites. Based on this criterion the consensus sequence 5' TTTT(N)_xAAAA (4 ≤ x ≤ 7), was derived that fitted all but one of the integration sites, the exception being Int 2 which contains only 3 dA residues. When this sequence was used to search the *H. influenzae* Rd genome, 167 potential Tn916 integration sites were identified. Since there are target sites, such as Int 2, which do not fit the consensus but have a secondary structure permissive for Tn916 integration, we assume

that the number of actual sites on the genome would be larger.

Of the Tn916 integration sites identified, 80 occurred in intergenic regions. This number was larger than expected since non-coding regions account for only 13% of total genomic DNA. This observation indicated that the distribution of Tn916 target sites on the Rd genome was biased. If the target sites had been randomly distributed then 145 of them would be expected to occur in ORFs. This disproportionate distribution of target sites was also reflected in the low frequency with which Tn916 insertions within ORFs were isolated experimentally. In general, intergenic regions of the *H. influenzae* genome have a higher A+T content than coding regions, which may reflect the fact that such regions harbour operator sequences. As a consequence, intergenic regions are therefore more likely to contain sequences which meet the criteria of the A+T-rich Tn916 target sites. The disruption of such regions may not, however, always result in a phenotype. The limited number of Tn916 integration sites in the genome of *H. influenzae*, in conjunction with their tendency to occur in non-coding regions, emphasizes the unsuitability of Tn916 as a tool for generalized mutagenesis of *H. influenzae*. It should be stressed, however, that the isolation of specific mutants using Tn916 is not impossible and that this system has been used to isolate mutations in iron regulatory genes (Holland *et al.*, 1992). The success of this strategy will, however, inevitably be dictated by whether genes of interest contain a Tn916 target site.

In summary we have demonstrated that the number sites at which Tn916 can integrate into the genome of *H. influenzae* is limited. Integration sites occur predominantly in intergenic regions and consequently may not always lead to an altered phenotype. It is therefore concluded that Tn916 is not suitable a tool for generalized mutagenesis of *H. influenzae*. In addition to the specific analysis of the Tn916 integration sites in *H. influenzae*, this study also demonstrates how bacterial genome sequences not only facilitate the rapid identification of genes of interest from small amounts of sequence information, but allow the efficiency of potential mutagenesis tools for the investigation of an individual organism to be assessed.

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REFERENCES

- Alexander, H. E. (1965). The *Haemophilus* group. In *Bacterial and Mycotic Infections of Man*, pp. 724–741. Edited by R. J. Dubos & J. G. Hirsch. London: Pitman Medical Publishing.
- Clewell, D. B., Flannagan, S. E. & Jaworski, D. D. (1995). Unconstrained bacterial promiscuity: the Tn916–Tn1545 family of conjugative transposons. *Trends Microbiol* 3, 229–236.

- Courvalin, P. & Carlier, C. (1987).** Tn1545. A conjugative shuttle transposon. *Mol Gen Genet* **206**, 259–264.
- DeSantis, P., Fua, M., Savino, M., Anselmi, C. & Bacchinofuso, G. (1996).** Sequence dependent circularisation of DNA: a physical model to predict the DNA sequence dependent propensity to circularisation and its changes in the presence of protein induced bending. *J Phys Chem* **100**, 9968–9976.
- Devereux, J., Haeberli, P. & Smithies, O. (1984).** A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.
- Fleischmann, R. D., Adams, M. D., White, O. & 37 other authors (1995).** Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496–512.
- Franke, A. E. & Clewell, D. B. (1981).** Evidence for a chromosome-borne resistance transposon (Tn916) in *Streptococcus faecalis* that is capable of ‘conjugal’ transfer in the absence of a conjugative plasmid. *J Bacteriol* **145**, 494–502.
- Gawron-Burke, C. & Clewell, D. B. (1984).** Regeneration of insertionally activated streptococcal DNA fragments after excision of Tn916 in *Escherichia coli*: strategy for targeting and cloning genes from Gram positive bacteria. *J Bacteriol* **159**, 214–221.
- Hallet, B., Rezsohazy, R., Mahillon, J. & Delcour, J. (1994).** IS231A insertion specificity: consensus sequence and DNA bending at the target site. *Mol Microbiol* **14**, 131–139.
- Herriot, R. M., Meyer, E. M. & Vogt, M. J. (1970).** Defined non-growth media for stage II development of competence in *Haemophilus influenzae*. *J Bacteriol* **101**, 517–524.
- High, N. J., Deadman, M. E., Hood, D. W. & Moxon, E. R. (1996).** The identification of novel lipopolysaccharide genes in *Haemophilus influenzae* using transposon Tn916 mutagenesis. *FEMS Microbiol Lett* **145**, 325–333.
- Holland, J., Towner, K. J. & Williams, P. (1992).** Tn916 insertion mutagenesis in *Escherichia coli* and *Haemophilus influenzae* type b following conjugative transfer. *J Gen Microbiol* **138**, 509–515.
- Kahler, C. M., Carlson, R. W., Rahman, M. M., Martin, L. E. & Stephens, D. S. (1996).** Inner core biosynthesis of lipooligosaccharide (LOS) in *Neisseria meningitidis* serogroup B: identification and role in LOS assembly of the α -1,2 N-acetylglucosamine transferase (RfaK). *J Bacteriol* **178**, 1265–1273.
- Kauc, L. & Goodgal, S. H. (1989).** Introduction of transposon Tn916 DNA into *Haemophilus influenzae* and *Haemophilus parainfluenzae*. *J Bacteriol* **171**, 6625–6628.
- Leidy, G., Hahn, E., Zamenhof, S. & Alexander, H. E. (1960).** Biochemical aspects of virulence of *Haemophilus influenzae*. *Ann N Y Acad Sci* **88**, 1195–1202.
- Lu, F. & Churchwood, G. (1995).** Tn916 target DNA sequences bind the C-terminal domain of integrase protein with different affinities that correlate with transposon insertion frequency. *J Bacteriol* **177**, 1938–1946.
- Sayle, R. A. & Milner-White, E. J. (1995).** RasMol: biomolecular graphics for all. *Trends Biochem Sci* **20**, 374.
- Scott, J. R., Bringel, F., Marra, D., van Alstine, G. & Rudy, C. K. (1994).** Conjugative transposition of Tn916: preferred target sites and evidence for conjugative transfer of a single strand and for a double-stranded circular intermediate. *Mol Microbiol* **11**, 1099–1108.
- Stephens, D. S., Swartley, J. S., Kathariou, S. & Morse, S. A. (1991).** Insertion of Tn916 in *Neisseria meningitidis* resulting in loss of Group B capsular polysaccharide. *Infect Immun* **59**, 4097–4102.
- Turk, D. C. (1984).** The pathogenicity of *Haemophilus influenzae*. *J Med Microbiol* **18**, 1–16.

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