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Site-Specific Recombination with the Chromosomal tRNA^{Leu} Gene by the Large Conjugative *Haemophilus* Resistance Plasmid

Ioanna D. Dimopoulou,† Joanne E. Russell,‡ Zaini Mohd-Zain, Rebecca Herbert, and Derrick W. Crook*

Interdepartmental Academic Unit of Infectious Diseases and Microbiology, Oxford University, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom

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Characterization of the sequences involved in recombination of the *Haemophilus* plasmid p1056 with the *Haemophilus influenzae* chromosome produced evidence indicating site-specific recombination with chromosomal tRNA^{Leu}. *attP* sequences identical to those of p1056 were found in six plasmids of diverse origin, suggesting that a family of *Haemophilus* plasmids recombines with chromosomal tRNA^{Leu}.

A β-lactamase-producing ampicillin-resistant (β-lact$^+$ Ap^r) strain of *Haemophilus influenzae* was first detected in the early 1970s (13). Thereafter β-lact$^+$ Ap^r or tetracycline, erythromycin, or chloramphenicol resistance rapidly increased simultaneously in many parts of the world (15). Not only has there been a dramatic increase in strains resistant to each of these antibiotics, but also individual strains resistant to multiple antibiotics have increasingly emerged (3, 12, 15). Many investigators have detected large transferable plasmids in these strains. It is generally accepted that these large resistance elements constitute a family of closely related plasmids and are distributed worldwide (2, 6).

Resistant clinical isolates seldom contain detectable extrachromosomal DNA (5, 20, 21), but following conjugation, recipients contain detectable extrachromosomal DNA with the features of plasmid rather than phage DNA (22). This suggested that plasmid is excised from the primary isolate and conjugally transfers to the recipient, where it replicates extrachromosomally (4, 5, 21). The molecular basis for these events was unknown. A 12-kb *Pst*I fragment containing the putative point of recircularization has been cloned (pB7) from a plasmid, p1056 (4, 11). This fragment also hybridized to two junction fragments in the parent strain, 1056 (4, 11). In this paper the sequence of the point of recircularization (*attP*) and the junction fragments consisting of the left and right attachment sites (*attL* and *attR*, respectively) in strain 1056 and the transconjugant JD 1056 were detected by PCR and directly sequenced. A further six strains from different parts of the world were also partly characterized by sequence analysis of *attP*.

Bacterial strains and plasmids and DNA purification. 1056 is a multiresistant (β-lact$^+$ Ap^r and tetracycline- and chloramphenicol-resistant) *H. influenzae* type b clinical isolate cultured in a case of meningitis (1). JD 1056 is the transconjugant derived from mating 1056 with nf38, a recombination-deficient derivative of *H. influenzae* Rd (18). The *H. influenzae* type b clinical strains 1876 (Ap^r), 1713 (Ap^r), 555 (Ap^r), 385 (Ap^r), and G268 (tetracycline resistant) originated from California, Alaska, India, Chile, and The Gambia, respectively. The NAD-requiring (V-dependent and hemin-independent) strain 10/10G was isolated in Oxford, United Kingdom. p1056 is the large conjugative resistance plasmid originating in 1056. pB7 contains the 12-kb *Pst*I fragment of p1056 and the putative site of recircularization cloned into pBluescript. Plasmid and chromosomal DNAs were purified using NucleoSpin Plasmid and NucleoSpin Tissue kits, respectively (Macherey-Nagel, Düren, Germany.)

Subcloning and sequencing of the site of recircularization. A 1.2-kb *Hin*dIII-*Xho*I fragment of pB7 containing the putative site of recircularization was subcloned into pBluescript and named pG6. Both strands were sequenced using M13 forward and reverse primers and by the design of further primers designated F1, R1, F2, and R2 (Table 1). The sequence of this fragment was analyzed using an ABI PRISM 377 sequencer (Applied Biosystems, Foster City, Calif.). The complete sequence was assembled using Staden (19) and Phred and Phrap (8–10) and analyzed with the GCG program (Genetics Computer Group Inc., Madison, Wis.). Related sequences were identified by comparison with the GenBank and EMBL databases. Sixty-six bases shared complete identity with those at the 3′ end of both copies of the *H. influenzae* Rd tRNA^{Leu}. The same 66 bp shared identity with the *attP* of HP1 (7, 23), a temperate phage of *H. influenzae*, but none of the remaining sequence of pG6 shared similarity to HP1. Sixty-four of the 66 bases shared identity with a virulence-associated locus in *Actinobacillus actinomycetemcomitans* (14).

PCR and sequence analysis of *attL*, *attR*, *attP*, and *attB*. It was postulated that p1056 recombined site specifically with one or both of the copies of *H. influenzae* Rd tRNA^{Leu}. An outer and inner pair of PCR primers targeted to the chromosomal sequences flanking both of the tRNA^{Leu} genes (referred to here as sites 1 and 2) were designed (Table 1). Further plasmid primers, JR3 and R1a, oriented towards *attL* and *attR*, respec-

* Corresponding author. Mailing address: Interdepartmental Academic Unit of Infectious Diseases and Microbiology, Oxford University, Level 7, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom. Phone: 44-1865-221226. Fax: 44-1865-764092. E-mail: derrick.crook@ndcls.ox.ac.uk.

† Present address: Medical School of Alexandroupolis, University of Thrace, Alexandroupolis, Greece.

‡ Present address: Laboratory for Physiology, Vrije Universiteit, 1081 BT Amsterdam, The Netherlands.

TABLE 1. PCR primers used for sequence analysis of *attP, attB, attI*, and *attR*

Primer	Nucleotide sequence	Basis or site
JR3	5′-GATAAGGGGATCAATTTCCCAC-3′	pG6[a] 264–285 bp
F1	5′-TTATCGTAATGTTTGTATCAGCCT-3′	pG6 295–318 bp
F2	5′-CGATCGTAGTGTATTGGCTAGGTAC-3′	pG6 673–697 bp
R1	5′-CCGGGAGCTTCTTCAGTTAAAGC-3′	pG6 1055–1077 bp[d]
R2	5′-GTACCTAGCCAATACACTACGATCG-3′	pG6 673–697 bp[d]
R1a	5′-AGCATAATAAGAGCTTAAGGTTGG-3′	pG6 1034–1057 bp[d]
X2	5′-CGCAAATGTTAGCACTAGGCG-3′	Site 1[b] 7457–7478 bp
X7	5′-TTATCTTACTTTATATCGCCGC-3′	Site 1 7521–7542 bp
Y8	5′-CTCAACGAAAGTAACTCATTGG-3′	Site 1 8248–8269 bp[d]
Y7	5′-GCGAAAGAAGTGATTCGTGAG-3′	Site 1 8212–8232 bp[d]
X5	5′-ACCACAATGACCATAAGCCG-3′	Site 2[c] 2091–2110 bp
X6	5′-CTGCGATTGCCTGCTTGAGC-3′	Site 2 2155–2174 bp
Y6	5′-TGGCCAAGAGTGGAGGAATTG-3′	Site 2 3395–3415 bp[d]
Y5	5′-TACGGGCTTACTTATTGAAGTC-3′	Site 2 3060–3081 bp[d]
Y4	5′-TAGATTGGATGGAGAGAATAGC-3′	Site 2 2936–2957 bp[d]

[a] Sequence from pG6 (this paper; accession no. U68467).
[b] Sequence obtained from segment 13 of the *H. influenzae* Rd genome (accession no. U32698).
[c] Sequence obtained from segment 9 of the *H. influenzae* Rd genome (accession no. U32694).
[d] Reverse complement.

tively, were designed (Table 1). *attL* and *attR* at site 1 were amplified with the primer pairs X2 plus R1 and Y8 plus JR3, respectively. The PCR products were directly sequenced using the inner PCR primers X7 plus R1a and Y7 plus F1, respectively. At site 2, PCR amplicons of *attL* and *attR* were generated using the primer pairs X5 plus R1 and Y6 plus JR3, respectively, and directly sequenced using the inner primer pairs X6 plus R1a and Y5 or Y4 plus F1, respectively. The sequences of *attL* and *attR* at both site 1 and site 2 revealed the presence of 66 bases of identity with the 3′end of chromosomal $tRNA^{Leu}$ (GenBank accession no. AF467991 to AF467994). Using PCR primers targeted to the plasmid recombined with the chromosome in the reverse orientation failed to generate PCR products, strongly suggesting a single orientation of integrative recombination. PCR of *attP* and *attB* (at both site 1 and site 2) in both 1056 and JD 1056 produced amplicons which on sequence analysis confirmed their respective identities. *attP* was amplified by PCR from plasmids of six strains derived from different parts of the world. The PCR products were directly sequenced and showed sequence identity to *attP* of p1056 (GenBank accession no. AF467995 to AF467800).

These observations are consistent with site-specific integrative and excisive recombination of the large resistance plasmid p1056 with chromosomal $tRNA^{Leu}$. The presence of *attP* sharing identity with p1056 in plasmids from strains originating from different parts of the world suggests that this family of plasmids recombines site specifically with chromosomal $tRNA^{Leu}$. The presence of these sequences in a V-dependent *Haemophilus* sp. supports the suggestion that this large plasmid also occurs in *Haemophilus parainfluenzae* (16, 17). The finding that extrachromosomal plasmid is seldom detectable by plasmid isolation or by hybridization of Southern blots in primary (clinical) isolates is explained by integrative recombination of plasmid with chromosomal $tRNA^{Leu}$. However, detection of *attP* or *attB* in 1056 (a clinical isolate) suggests that there is low-frequency excisive recombination of plasmid in this isolate. The extrachromosomal site of plasmid in transconjugants is supported by ready isolation of closed circular plasmid. However, integrative recombination of plasmid in the transconjugant JD 1056 is revealed by detection of *attL* and *attR* at both $tRNA^{Leu}$ loci.

Nucleotide sequence accession number. Sequences have been lodged with GenBank under accession no. U68467 and AF467995 to AF467800.

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