Molecular Organization of Small Plasmids Bearing \textit{bla}_{TEM-1} and Conferring Resistance to beta-lactams in \textit{Haemophilus influenzae}

Running title: Small resistance plasmids in \textit{H. influenzae}

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Abstract:

TEM-1 is the dominant β-lactamase of *Haemophilus influenzae* and can be located on small plasmids.

Three distinct plasmids from 4,304 to 5,646 nt were characterized: pA1606, pA1209 and pPN223. In addition to TEM-1 and a replication enzyme of the Rep 3 superfamily, pA1606 encodes a Tn3 resolvase gene and pA1606 and pA1209 encode an ORF similar to a plasmid recombination enzyme gene described in Gram-positive bacteria. The plasmids transformed strain Rd to the ampicillin-resistant phenotype.
The genus *Haemophilus* comprises small, Gram-negative rods that colonize mucosal surfaces of humans and animals (7). *Haemophilus influenzae* is the major human pathogen of the genus and a significant cause of respiratory tract infections, acute otitis media, pneumonia and purulent meningitis (17). The prevalent resistance mechanism in *H. influenzae* is the production of narrow spectrum β-lactamases, which hydrolyze the penicillin class of β-lactams. Two different types of β-lactamases have been described in *H. influenzae*, TEM-1 and ROB-1, the former being by far the most common (1).

Recently, the location of the ROB-1 gene on a small plasmid designated pB1000 in *Haemophilus parasuis* was described (12); subsequently, identical plasmids were documented in *Pasteurella multocida* and *Haemophilus influenzae* from Europe, Australia and North America (14, 16), demonstrating an interspecies dissemination involving various animal and human hosts. The pB1000 plasmid is a mobile replicon belonging to the ColE1 superfamily and the MOB_HEN family (2, 3). In contrast, the *bla* _TEM-1_ gene is usually located on large (40 kb) integrative conjugative elements (ICEs) when present in *H. influenzae*, and less commonly on small (<10 kb) non-conjugative plasmids (8). Detailed characterization of these small *bla* _TEM-1_ bearing plasmids has not yet been published.

Four hundred and seventy clinical isolates of *H. influenzae* were previously characterized for mutational resistance caused by amino acid substitutions in penicillin-binding protein 3 (10); 98 β-lactamase-producing strains from that collection are addressed in this study. All strains were positive for *bla* _TEM-1_ and negative for *bla* _ROB-1_, as evaluated by PCR using primers targeting *bla* _TEM-1_ and *bla* _ROB-1_ (5, 12) (#1-4, Table 1).

To search for TEM-1-associated genetic elements, two separate PCR were performed targeting the orf-51 region of ICEhin1056 (11), and the replication gene (*rep*) of small plasmids (unpublished primers kindly provided by S. G. Tristram), respectively (#5-8, Table 1). Eighty β-lactamase-producing strains were positive for ICEhin1056 only, 17 were positive for *rep* only, and one strain was positive for both. A PCR spanning the TEM-1 and REP genes (Fig. 1) gave rise to amplicons.
except with the strain that was positive for both ICE and rep. The REP plasmid of this strain was fully sequenced (data not shown) and no TEM-1 gene was found, indicating that in this strain \( \text{bla}_{\text{TEM-1}} \) 53 is located only on the ICE.

Plasmid preparations (mini prep kit, Qiagen, Hilden, Germany) revealed the presence of plasmids in the 4-10 kb range in ICE-negative strains. To identify the plasmids conferring \( \beta \)-lactam resistance, we performed inverse PCR of the TEM-1 gene (#11-12, Table 1) using a Phusion polymerase (Finnzymes, Woburn, MA, USA) according to manufacturer’s instructions. Different \( \text{bla}_{\text{TEM-1}} \) bearing plasmids in the range of 4-6 kb were detected in the ICE-negative strains. A representative of the largest \( \text{bla}_{\text{TEM-1}} \) bearing plasmids was completely sequenced by primer walking (primers available on request). The plasmid, designated pA1606, is schematically presented in Fig 1A and encodes four open reading frames (ORFs): i) a \( \text{bla}_{\text{TEM-1}} \) gene of 861 nt with 100 % identity to several homologues deposited in GenBank; ii) an ORF encoding a 330 aa protein with 60-75 % similarity with replication enzymes (REP) of the Rep 3 superfamily, previously described from small resistance plasmids in other members of \( \text{Pasteurellaceae} \) (6, 13); iii) an ORF encoding a 423 aa hypothetical protein of which the first 197 aa of the NH3 part showed 45% identity with a plasmid recombination enzyme (PRE) of the Mob-Pre superfamily described in Gram-positive bacteria (4); and iv) a Tn3 resolvase gene (\( \text{tnpR} \)) with 100 % identity to TnpR genes in large plasmids from Gram-negative bacteria (9).

Finally, an ORF encoding a 151 aa truncated protein, of which the first 104 aa showed 100% similarity to Tn3 transposase (TnpA) proteins (18), was found in pA1606.

The four genes, \( \text{rep} \), \( \text{bla}_{\text{TEM-1}} \), \( \text{pre} \), and \( \text{tnpR} \), were mapped in the 17 \( \text{bla}_{\text{TEM-1}} \)-\text{rep}-positive strains using primers listed in Table 1 (#3-4, and #7-18). Three strains were positive for all four genes, eleven strains were positive for \( \text{rep} \), \( \text{bla}_{\text{TEM-1}} \), and \( \text{pre} \), while three strains carried plasmids positive for \( \text{rep} \) and \( \text{bla}_{\text{TEM-1}} \) only. All three \( \text{bla}_{\text{TEM-1}} \) plasmid types were disseminated into non-clonally related strains as evaluated by pulsed-field gel electrophoresis of genomic DNA after cleavage with \( \text{smaI} \) (Roche Diagnostics) (data not shown). A representative from each of the two latter plasmid types was also...
sequenced (pA1209 and pPN223, respectively, Fig. 1B and 1C). While pA1209 appeared to be a truncated version of pA1606, pPN223 also contained additional genetic material, namely a larger linker between blaTEM-1 and rep, and an ORF encoding a hypothetical protein of 166 aa approximately 500 nt upstream of blaTEM-1. Moreover, a sequence with homology to the promoter sequence of ROB in the pB1000 plasmid was located approximately 1300 nt upstream of blaTEM-1 in pPN223. This explains the aberrant results of a multiplex tem-rob PCR (15) when used on strains containing pPN223 found in a previous study (10): the forward primer from the flanking region of blaROB-1 and the internal reverse primer of blaTEM-1 combined to produce a PCR product of a size that erroneously could suggest the presence of blaROB-1. All three plasmid types contained two conserved regions (gray boxes in Fig. 1), a 284 nt region immediately upstream from rep, and a 122 nt region approximately 80 nt upstream from pre in pA1209 and pA1606, or 140 nt upstream from ORF in pPN223 (the ORF of pPN223 encoding a hypothetical protein is not shown in Fig. 1). Primers #19-20 and #21-22 (Table 1) were designed to hybridize with the conserved regions and were successfully used to amplify products from all 17 strains with blaTEM-1–bearing small plasmids.

pA1606, pA1209, and pPN223 were transformed into the transformation-competent strain Rd by electroporation. The transformants were selected by subculture on chocolate agar containing 1 µg/ml ampicillin. Donor strains and Rd transformants were subjected to antibiotic susceptibility testing using a commercial microbroth dilution system (Sensititre). As expected, the transformants were resistant to ampicillin, but susceptible to amoxicillin and piperacillin in the presence of β-lactamase inhibitors (Table 2). Comparison of transformants with strain Rd by PFGE, and plasmid extraction from transformants, documented successful transformation.

In conclusion, our data show that clinical isolates of H. influenzae may harbor at least three different types of small plasmids encoding a replication enzyme of the REP 3 superfamily and a TEM-1 β-lactamase. All of these plasmids confer resistance to ampicillin. The plasmids do not encode conjugative machinery, mob genes, or the H. influenzae DNA signal sequence (USS). Nevertheless,
the presence of the same plasmid type in genetically distinct strains of *H. influenzae* show that horizontal transfer of these plasmids occurs in nature.

**Nucleotide Sequence Accession Numbers**

Nucleotide sequences from this study have been deposited in GenBank under accession numbers JQ611726 (pA1606), JQ783055 (pA1209) and JQ611727 (pPN223).
Acknowledgements

The authors are grateful to Professor Steven G. Tristram, School of Human Life Sciences, University of Tasmania, Launceston, Tasmania, Australia, for helpful advice and description of rep PCR.
REFERENCES


15. Tristram, S. G., and S. Nichols. 2006. A multiplex PCR for beta-lactamase genes of

16. Tristram, S. G., R. Littlejohn, and R. S. Bradbury. 2010. blaROB-1 presence on pB1000 in
Haemophilus influenzae is widespread, and variable cefaclor resistance is associated with altered
10.


2009. Complete nucleotide sequence of pCTX-M360, an intermediate plasmid between pEL60 and
Figure 1. Schematic representation of small plasmids bearing $bla_{\text{TEM-1}}$ in *H. influenzae*. Genes are represented by white arrows; conserved regions are shown as gray boxes. *rep*: replication gene; $bla_{\text{TEM-1}}$: β-lactamase TEM-1 gene; *pre*: plasmid recombination enzyme gene; *tnpR*: transposon Tn3 resolvase gene.
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17 tnpR 145F GATTTGCTGAGGATGAAGGTG pA1606 3050R
18 tnpR 409R CTTTCAGCTTTGCTTCTGTC pA1606 2786F
19 Cons.-1 F GAAAGCCCAAAAGAGCCGAAAG pPN223 14F
20 Cons.-1 R CTTTCGGCTCTTTTGGGCTTTC pPN223 35R
21 Cons.-2 F GCGTTAAATTGGCGTAGCCTG pPN223 2843F
22 Cons.-2 R CAGGCTACGCAATTTAAACGC pPN223 2863R

GenBank Accession numbers: pB1000, DQ840517; ICEhin1056, AJ627386; pA1606, JQ611726; pPN223, JQ611727.
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<th>TZP</th>
<th>CXM</th>
<th>CRO</th>
<th>LVX</th>
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<td>1</td>
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<td>≤0.008</td>
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</table>

¹ AMP, ampicillin; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CXM, cefuroxime; CRO, ceftriaxone; LVX, levofloxacin.