TEM-1-encoding small plasmids impose dissimilar fitness costs on *Haemophilus influenzae* and *Haemophilus parainfluenzae*

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INTRODUCTION

Resistance to beta-lactam antimicrobial agents in *Haemophilus influenzae* is commonly mediated by production of beta-lactamases. Only two beta-lactamases, TEM-1 and ROB-1, have been described for *H. influenzae*, with TEM-1 being by far the more prevalent (95%) of the two (Farrell et al., 2005). Four different TEM beta-lactamases (TEM-1, TEM-15, TEM-34 and TEM-182) have been documented in *H. parainfluenzae* (Tristram et al., 2008; García-Cobos et al., 2013).

In *H. influenzae*, TEM-1 is primarily encoded on large integrative and conjugative elements (Leaves et al., 2000), and less commonly (14–17%) on small plasmids (Søndergaard et al., 2012; Tristram et al., 2012; Fleury et al., 2014). Six different *bla*<sub>TEM-1</sub>-bearing plasmids of 4–6 kb have been characterized (Søndergaard et al., 2012; Tristram et al., 2012). These plasmids can be separated into three groups: the two-gene plasmids encode *bla*<sub>TEM-1</sub> and *rep*, the three-gene plasmids also encode *mob/pre*, and the four-gene plasmids also encode *tnpR*. The two-gene group is only represented by pPN223 (4304 bp), which also has a distinct backbone sequence. The three-gene group encompasses pLF55 and pA1209 (5142 bp), while the four-gene group encompasses pLFH49, pLFH64 and pA1606 (5646 bp). Transcription of TEM-1 is regulated by two different promoters, namely Pa/Pb (pPN223 and pLFH49), and Prpt (pLF55, pA1209, pLFH64 and pA1606).

The plasmid encoding ROB-1 in *H. influenzae* is designated pB1000 and is 4.6 kb in size (San Millan et al., 2010). pB1000 has been detected in other Pasteurellaceae species of animal origin (San Millan et al., 2007, 2009, 2010; Tristram et al., 2010), but not in *H. parainfluenzae*. In *H. parainfluenzae* *bla*<sup>TEM-15</sup> and *bla*<sup>TEM-34</sup> have been found on small plasmids (3.7 kb and 5.5 kb, respectively), whereas *bla*<sup>TEM-1</sup> and *bla*<sup>TEM-182</sup> have been described on large integrative and conjugative elements (Tristram et al., 2008; García-Cobos et al., 2013). Plasmid pSF3 encoding TEM-15 (an extended spectrum beta-lactamase – ESBL)
has not been fully sequenced, but TEM-15 is under control of the Prpt promoter. A plasmid bearing TEM-34 was not available for this investigation; the reported plasmid (p72322) is identical to pLFH64, except for the single amino acid substitution in TEM that renders the beta-lactamase resistant to clavulanic acid (García-Cobos et al., 2013).

pB1000 has a competitive disadvantage of approximately 9% per ten generations relative to the parental strain when transformed into H. influenzae strain Rd (San Millan et al., 2010). Fitness cost analyses of blaTEM-bearing plasmids have not, to our knowledge, been performed. To aid our understanding of the epidemiology of small beta-lactamase-bearing plasmids, we assessed stability and fitness cost, and performed expression analysis after introduction of TEM-1-, TEM-15- and ROB-1-bearing plasmids into recipient strains of H. influenzae and H. parainfluenzae.

**METHODS**

**Bacterial strains, culture conditions and susceptibility testing.** The characteristics of the strains and plasmids used in this study are listed in Table 1.

Ampicillin-susceptible H. influenzae strain Rd (Fleischmann et al., 1995) and H. parainfluenzae strain HK 23 (CCUG 49489) (Hedegaard et al., 2001) were used as transformation recipients. Small beta-lactamase bearing plasmids were isolated from strains of H. influenzae (pPN223, pA1209 and pA1606) (Søndergaard et al., 2012) or H. parainfluenzae (pSF3) (Tristram et al., 2008). Plasmid pB1000 was kindly donated by Professor Bruno Gonzales-Zorn (San Millan et al., 2010). The blaTEM plasmids used in this study represent each of the three TEM-1-bearing small plasmid types hitherto characterized in H. influenzae.

**Plasmid extraction and transformation.** Plasmid DNA was extracted using a QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer’s instructions.

Electrocompetent cells were prepared from H. influenzae strain Rd and H. parainfluenzae strain HK 23 and transformed with purified plasmid DNA using electroporation as described elsewhere (Ubukata et al., 2001). Transformants were selected on chocolate agar supplemented with 4 μg ampicillin (AMP) ml⁻¹. Electroporated controls with no added DNA were plated on selective agar to assess the selective quality of the agar. Selected colonies from each transformation experiment were subcultured twice on AMP-containing agar. Production of beta-lactamase was confirmed using nitrocefin dry slides (BBL DrySlide Nitrocefin; Becton, Dickinson and Company).

**Growth kinetics, plasmid curing and competition experiments.** The fitness cost of each plasmid was assessed by growth kinetics, plasmid curing and competition between recipient strain and transformant. A saline suspension with an optical density of 1.0 McFarland was prepared for each strain or transformant and used for the simultaneous execution of the three assays. The assays were repeated on four separate occasions.

For measurement of growth kinetics, the saline suspensions were diluted 1000 times in sBHI, and 200 μl was added to wells in triplicate in a 96-well flat bottomed plate. Absorbance at 620 nm was measured every 10 min for 24 h on a microplate spectrophotometer (Thermo Scientific Multiskan GO Microplate Spectrophotometer) set on 37°C and 1 min of shaking every other minute.

To determine the stability of the plasmids, saline suspensions of H. influenzae strain Rd and H. parainfluenzae strain HK 23

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**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description and characteristics</th>
<th>Reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>Rd KW20</td>
<td>Amp H. influenzae recipient strain</td>
<td>Fleischmann et al. (1995)</td>
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<tr>
<td>HK23 (CCUG 49489)</td>
<td>Amp H. parainfluenzae recipient strain</td>
<td>Hedegaard et al. (2001)</td>
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<tr>
<td>Rd/pPN223</td>
<td>Rd KW20 transformed with pPN223</td>
<td>Søndergaard et al. (2012)</td>
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<td>Rd/pA1209</td>
<td>Rd KW20 transformed with pA1209</td>
<td>Søndergaard et al. (2012)</td>
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<td>Rd/pA1606</td>
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<td>Søndergaard et al. (2012)</td>
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<td><strong>Plasmids</strong></td>
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<td>pPN223</td>
<td>rep, blaTEM-1 isolated from H. influenzae</td>
<td>Søndergaard et al. (2012)</td>
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<tr>
<td>pA1209</td>
<td>rep, blaTEM-1, mob/pre isolated from H. influenzae</td>
<td>Søndergaard et al. (2012)</td>
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<td>pA1606</td>
<td>rep, blaTEM-1, mob/pre, tnpR isolated from H. influenzae</td>
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<tr>
<td>pB1000</td>
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<td>San Millan et al. (2010)</td>
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transformed with each plasmid were diluted 1000 times in sBHI and incubated at 37 °C with continuous shaking (130 r.p.m.). Every 24 h for 5 days, the suspension was diluted 1000 times in fresh antibiotic-free sBHI, and aliquots were plated on nonselective chocolate agar. The proportion of colonies expressing beta-lactamase was assessed by replica plating of 50 colonies on chocolate agar plates containing 4 μg AMP ml⁻¹.

For competition experiments, 5 μl of the saline suspension of *H. influenzae* strain Rd or *H. parainfluenzae* strain HK 23 were mixed with equal amounts of the same strain transformed with the plasmid in question in 5 ml of antibiotic-free sBHI. The mixture was incubated at 37 °C with continuous shaking (130 r.p.m.). Every 24 h for 5 days, a total of 5 μl of the mixture were diluted 1000 times in fresh sBHI, and aliquots were plated on nonselective chocolate agar. The proportion of colonies expressing beta-lactamase was assessed by replica plating of 100 colonies on chocolate agar plates containing 4 μg AMP ml⁻¹.

**Plasmid copy number and beta-lactamase expression.** Strains were grown in sBHI to an OD₆₂₀ of 0.2–0.3 at 37 °C and 130 r.p.m. Five millilitres of suspension was centrifuged and the pellet was resuspended in 1 ml RNAprotect Bacterial reagent (Qiagen) and kept at −20 °C until further use. RNA and DNA were extracted from the suspension using a MagNA Pure Compact instrument using a MagNA Pure Compact Nucleic Acid Isolation kit (Large Volumen) (Roche). The samples were split into an RNA sample for preparation of cDNA, and a DNA sample for assessment of the plasmid copy number. Residual DNA was degraded from the RNA sample using a Turbo DNA free kit (Ambion/Life Technologies) and 50 % more DNase than recommended by the manufacturer in a Veriti 96-well Thermal Cycler (Applied Biosystems) for 30 min at 37 °C, and 5 min at 95 °C. cDNA was prepared with TaqMan Reverse Transcription reagents (Life Technologies) in a Veriti 96-well Thermo Cycler for 10 min at 25 °C, 30 min at 48 °C and 5 min at 95 °C. The cDNA and purified DNA were mixed with primers, probes, and TaqMan Fast Advanced Master Mix (Life Technologies) and quantitative PCR was run in triplicates in a LightCycler 480 (Roche). RNA reverse transcriptase served as a negative control. Relative gene expression and plasmid copy number were quantified with LightCycler Relative Quantification software (Roche Applied Science) and normalized to the single-copy housekeeping genes *adk, fucK* and *mdh* (*H. influenzae*), or *adk, mdh* and *recC* (*H. parainfluenzae*). The PCRs were designed to have similar amplification efficiency (primers and probes are listed in Table S1, available in the online Supplementary Material).

**RESULTS AND DISCUSSION**

**Fitness cost of small beta-lactamase-encoding plasmids in *H. influenzae* and *H. parainfluenzae***

None of the plasmids appeared to depress the growth rate of *H. influenzae* strain Rd (Fig. 1a). For *H. parainfluenzae* strain HK 23, plasmids pA1209 and pPN223 did not affect the growth kinetics, while plasmids pA1606, pSF3 and pB1000 reduced both the growth rate and the maximal cell density at stationary phase assessed after 24 h (Fig. 1b).

Competition assays between recipient and transformant can reveal subtle disadvantages that are not readily disclosed by simple growth curve measurements (Andersson & Hughes, 2010). Equal numbers of bacteria with and without plasmid were mixed and subcultured every 24 h for 5 days, and the proportion of bacteria carrying the plasmid at each time point was monitored. Fig. 2 shows a significant cost imposed on both *H. influenzae* and *H. parainfluenzae* by TEM-15 (pSF3)- and ROB-1 (pB1000)-bearing plasmids: after only 24 h, *H. parainfluenzae* strain HK 23 harbouring plasmids pB1000 or pSF3 was almost completely outnumbered by the parental strain without plasmid, while the effects on *H. influenzae* were more protracted (Fig. 2g–j). The competitive disadvantage of plasmid pSF3 on *H. parainfluenzae* strain HK 23 may partly explain why TEM-15, an ESBL, has only sporadically been reported in this species (Tristram et al. 2008). However, it is a matter of concern that the fitness cost of this replicon is lower in the more pathogenic *H. influenzae* (Fig. 2g) than in its theoretical native host (Fig. 2h). The competitive disadvantage of plasmid pB1000 on *H. influenzae* strain Rd found in this study is in agreement with that reported by San Millan et al. (2010). In contrast, the three TEM-1-bearing plasmids were differentially tolerated by the two bacterial hosts: pPN223 and pA1606 did not impose a significant fitness cost to *H. influenzae* strain Rd, and pA1209 did not impose a significant fitness cost to *H. parainfluenzae* strain HK 23, while the reverse combinations resulted in significant disadvantages (Fig. 2a–f). The initial fitness

![Fig. 1. 24 h growth curves of (a) *H. influenzae* strain Rd isogenic transformants, and (b) *H. parainfluenzae* HK 23 isogenic transformants. The curves represent the mean of three independent experiments.](image-url)
Fitness cost of small beta-lactamase plasmids

(a) Rd/pPN223
(b) Rd/pA1209
(c) Rd/pA1606
(d) HK23/pPN223
(e) HK23/pA1209
(f) HK23/pA1606
(g) Rd/pSF3
(h) HK23/pSF3
(i) Rd/pB1000
(j) HK23/pB1000
cost imposed by pA1606 on *H. influenzae* was not detectable after 5 days (Fig. 2e). We did not sequence the plasmids at the end of the experiment, and mutations leading to a decrease in fitness cost cannot be excluded. However, the effect was similar in four separate experiments. Another possible mechanism of amelioration of fitness cost of newly introduced plasmids is reduction of plasmid copy number (San Millan et al., 2015).

A recent study of *H. influenzae* AMP-resistance in Sweden found that 16% of 278 beta-lactamase expressing isolates carried TEM-1 on small plasmids. Of the 44 small TEM-1-bearing plasmids, four were pPN223, 19 were similar to A1209, while 21 were similar to pA1606 (Fleury et al., 2014). The low fitness cost of pA1606 could be implicated in the high prevalence of this plasmid type, while the low prevalence of pPN223 could be related to its lack of the *mobl/pre* mobilization gene. However, our fitness cost analysis does not give any clues to the high prevalence of pA1209 in *H. influenzae* reported in that study.

The plasmid curing assay measures the stability of plasmids within the host cells. This was performed by subculturing the transformants in non-selective broth every 24 h for 5 days and assessing the proportion of cells that had lost the plasmid and become susceptible to AMP. Curing was only observed for *H. parainfluenzae* strain HK 23 harbouring the ROB-1-encoding plasmid pB1000. After 5 days, 76 ± 12% (N=3; 31–44 colonies out of 50 replicate plate colonies) of the HK 23/pB1000 strains had lost pB1000. pB1000 has been detected in several *Pasteurellaceae* species (San Millan et al., 2007, 2009, 2010; Tristram et al., 2010), but not in *H. parainfluenzae*. The imposed fitness cost and the relatively fast curing of pB1000 may explain the absence of ROB-1 in *H. parainfluenzae*.

### Plasmid copy number and beta-lactamase expression in relation to fitness cost of small beta-lactamase-encoding plasmids

Synthesis of plasmid copies, mRNA and encoded proteins impose an energetic burden, which may lead to a fitness cost for the recipient cell (Lenski, 1998). Therefore, plasmid copy number and beta-lactamase mRNA expression were assessed for the five plasmids in *H. influenzae* strain Rd and *H. parainfluenzae* strain HK 23.

Levels of beta-lactamase mRNA (normalized to the expression of three housekeeping genes) are presented in Fig. 3(a). The results show that TEM-15 and ROB-1 were expressed at a higher level than TEM-1 in both species. Variation in *blaTEM* promoters has previously been reported and could affect TEM expression (Tristram et al., 2007, 2012). Plasmids pA1606 and pA1209 carry the *Prpt* promoter, whereas pPN223 carries the *Pa/Pb* promoter. Variation in promoter sequences did not affect transcription levels of TEM-1 (Fig. 3a).
Plasmid quantification (normalized to single-copy housekeeping genes) showed a generally lower plasmid copy number per cell in *H. parainfluenzae* strain HK 23 compared to *H. influenzae* strain Rd (Fig. 3b). The plasmid copy number of pA1209 was much lower in both species, while pA1606, pSF3 and pB1000 were present in the highest copy numbers per cell (Fig. 3b).

High beta-lactamase expression in transformants harbouring pSF3 and pB1000 could contribute to the increased fitness cost observed for these plasmids. However, the level of mRNA expression and plasmid copy number of the three TEM-1-encoding plasmids did not reflect the different profiles of fitness cost observed for the two bacterial hosts.

In conclusion, we show that small plasmids encoding TEM beta-lactamases isolated from *H. influenzae* and *H. parainfluenzae* can be stably transferred to recipient strains of the other species. TEM-1-encoding plasmids are associated with the lowest level of fitness cost in both species, but different TEM-1 plasmids confer different burden on the two hosts. The results are corroborated by the predominance of the TEM-1 beta-lactamase in *H. influenzae*, while more studies on the prevalence and type of small resistance plasmids in *H. parainfluenzae* are needed.

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REFERENCES


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