Plasmid-encoded functions compensate for the biological cost of AmpC overexpression in a clinical isolate of Salmonella typhimurium

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Introduction

AmpC β-lactamases are cephalosporinases that may be either chromosomal or plasmid-encoded in Gram-negative organisms. Plasmid-encoded ampC genes are considered to have originated from the inducible, chromosomal ampC of organisms such as Enterobacter spp., Citrobacter freundii, Morganella morgani, Hafnia alvei and Aeromonas spp. More than 50 different ampC genes have been reported in clinical isolates worldwide including over 20 plasmid-borne ampC genes. Organisms producing AmpC β-lactamases can be resistant to all β-lactam antibiotics except cephalime, cepirome and carbapenems. AmpR encodes for a transcriptional regulator of ampC. It is located upstream of ampC and is divergently transcribed. In the presence of certain cofactors, AmpR can act as a repressor of ampC expression or as an activator upon addition of certain β-lactams or other stimuli.

Plasmid-encoded ampC genes may or may not be associated with ampR. For example, only blaACT,5 blaDHA,6 and blaDHA-2 have been shown to be associated with ampR.

Salmonella spp. are one of the most virulent members of the Enterobacteriaceae family. Virulence of a pathogen can be enhanced by either gain of function due to acquisition of virulence genes or loss of function due to deletion of chromosomal genes. In both S. dysenteriae and S. flexneri, the ‘black hole’ included the ampC β-lactamase gene. Acquisition of drug resistance by a pathogen can lead to fitness loss such as reduced growth and virulence. Therefore, the question becomes whether acquisition of ampC by Salmonella leads to biolog-

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ical cost such as reduced growth rate or virulence. Morosini et al. addressed this question by mobilizing plasmids which contained the cloned chromosomal ampC and ampR together or the ampC alone from Enterobacter cloacae into Salmonella typhimurium. They reported that acquisition of a plasmid expressing ampC alone by Salmonella was associated with a biological cost indicated by reduced growth rate in laboratory medium and reduced capacity to invade mammalian cells. Furthermore, the authors reported that simultaneous acquisition of ampC–ampR was a requirement for keeping ampC expression at low constitutive levels, which allowed Salmonella to overcome the deleterious effect of high-level ampC expression.

Our laboratory recently described S. typhimurium strain 100, isolated from a patient with gastroenteritis that produced three different β-lactamases, OXA-30, SHV-9 and CMY-7.17 CMY-7 belongs to a family of plasmid-encoded AmpC enzymes and is 93% similar to the β-lactamase of C. freundii –blaCMY-7 expression on growth and virulence of S. typhimurium strain 100.

Materials and methods

Bacterial strains and plasmids

S. typhimurium strain 100 has been previously described.17 C. freundii 21 is a clinical isolate which has an inducible chromosomal ampC, whereas C. freundii 21 M is a derepressed (clinically non-inducible) mutant derived from C. freundii 21.18 S. typhimurium strain LT2 was a gift from Dr Diana Jones (University of Wisconsin–Madison). Sodium azide-resistant Escherichia coli J53Az was kindly supplied by Dr George Jacoby (Lahey Clinic, Burlington, MA, USA). pCMY-7 designates the plasmid within S. typhimurium 100 which encodes blaCMY-7. pPAH1 designates the plasmid encoding the blaCMY-7 structural gene and 496 bp of the upstream region cloned into a high-copy number (>100 copies per cell) cloning vector, pCR-TOPO-XL, a pUC 19 derived vector (Invitrogen, Carlsbad, CA, USA). S. typhimurium LT2:pCMY-7 is the Salmonella transconjugant harbouring pCMY-7 and LT2:pPAH1 designates the Salmonella LT2 transformant harbouring pPAH1.

Polymerase chain reaction (PCR) and DNA sequencing

DNA template was prepared from S. typhimurium strains and PCR was carried out as previously described.17 Primers used in this study are listed in Table 1. PCR products were sequenced directly after treating with ExoSap (USB, Columbus, OH, USA) to remove unwanted nucleotides and primers. PCR amplicons were sequenced on a DNA stretch sequencer from Applied Biosystems (Foster City, CA, USA). Sequence analyses were carried out online using the BLAST program (www.ncbi.nlm.nih.gov).

Plasmid analysis and plasmid copy number determination

Plasmid profiles were determined as previously described.17 The relative copy number of pCMY-7 was determined by a PCR-based methodology using primers CMY7F and CMY7R (Table 1) as previously described.17 Primers SalAmpDF1 and SalAmpDR1 (Table 1) were used for amplification of ampD of S. typhimurium strain 100, which was used as a single copy gene reference for copy number determination.

Upstream sequence determination of blaCMY-7

Genome Walker (Clontech, Palo Alto, CA, USA), a PCR-based methodology that permits amplification of unknown DNA sequence adjacent to known sequence was used to amplify the upstream region of the gene. Gene specific primers CMY7GSP1 and CMY7GSP2 (Table 1) were used in addition to adapters supplied by the manufacturer to generate a Genome Walker library. An upstream fragment of 524 bp was generated and sequenced with primers CMYUPF1 and CMYGSP1.

Primer extension analysis

RNA was isolated using Trizol-Max (Invitrogen). Primers (CMYPE and CMY2PE for blaCMY-7, and chromosomal ampC of C. freundii, respectively) (Table 1) were annealed to 20 µg of total RNA at 50°C and primer extension was carried out using 100 U of MuLV reverse transcriptase (Perkin-Elmer, Norwalk, CT, USA) as previously described.3 One microgram of RNA was used for 16S rRNA reactions with primer B. Extension products were visualized by exposing the gel to a storage

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Purpose</th>
<th>GenBank accession number</th>
</tr>
</thead>
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<td>CGGCAGCAATGTTGAGAAAGGTCGCTAGC</td>
<td>GW</td>
<td>X91840</td>
</tr>
<tr>
<td>CMY7GSP2</td>
<td>CGGTAGTGGTGCGATATCCGTCGTCGATCG</td>
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</tr>
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<td>X91840</td>
</tr>
<tr>
<td>CMY2PE</td>
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<td>X91840</td>
</tr>
<tr>
<td>Primer B</td>
<td>CCAGACATATACTCCGCCTCC</td>
<td>PE</td>
<td>Y17669</td>
</tr>
</tbody>
</table>

GW, genome walking; Seq, sequencing; Clon, cloning; CN, copy number; PE, primer extension.

<sup>a</sup>Sequence of the primer, 5′-3′.
phosphor (Eastman Kodak Co., Rochester, NY, USA) for 2 days. Quantification of bands was carried out using ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA, USA).

**Conjugation experiments**

Conjugation experiments were carried out by a liquid mating procedure. Initially, *S. typhimurium* 100 plasmids were transferred to the sodium azide-resistant *E. coli*, J53Az8, with Luria–Bertani (LB) agar containing 150 mg/L sodium azide (Sigma, St Louis, MO, USA) and 50 mg/L ampicillin (Sigma) as the selection medium. The plasmid containing blaCMY-7 was then conjugated from the azide-resistant *E. coli* J53Az8 to *S. typhimurium* strain LT2 and the transconjugant was designated LT2:pCMY-7. Chromagar Salmonella (Chromagar, Paris, France) was used to differentiate between *E. coli* (blue) and *Salmonella* (pink) colonies. The transconjugants were screened for the presence of blaCMY-7 by PCR using primers CMY2SF1 and CMY2DR1 (Table 1) as described above following each mobilization step.

**Cloning of blaCMY-7 and the upstream region**

The blaCMY-7 and 493 bp upstream region was amplified by PCR as described above using primers CMYUPF1 and CMY2DR1 (Table 1). The PCR product was purified using a SNAP column, ligated into pCR-TOPO-XL cloning vector and transformed into *E. coli* Top10 competent cells (Invitrogen). The resulting plasmid (pAH1) was isolated and the sequence was generated using Genome Walker methodology. The PCR-generated insert was verified using primers CMYUPF1, CMY25F1, CMY7GSP2 and CMY2DR1 (Table 1).

**Growth curves**

Overnight cultures of *S. typhimurium* strains were grown in 5 mL of LB (Difco, Sparks, MD, USA) medium and 1 mL was used to inoculate 100 mL of LB medium. The cultures were grown with good aeration in an orbital shaker (140 rpm) at 37°C for 10 h. During this time, aliquots of culture were removed hourly for measurement of cell density (i.e. the OD600) and determination of viable counts.

**Antibiotic susceptibility testing**

Cefoxitin MICs for *S. typhimurium* strains 100, LT2, LT2:pCMY-7 and LT2:pAH1 were determined by Etest according to the National Committee for Clinical Laboratory Standards (NCCLS) protocol.

**Disc induction assay**

Disc induction assays were carried out as previously described using cefoxitin (30 µg) and cefotaxime (30 µg) placed 13 mm apart on Mueller–Hinton agar plates.

**Invasion assay**

In vitro invasion assays using mammalian gastric carcinoma tissue culture cells (ATCC CRL/1739) were carried out with a multiplicity of infection (MOI) of 20 as previously described. The invaded bacteria were released by lysing the mammalian cells with 1×PBS–0.1% Triton X-100, and the number of intracellular bacteria (cfu) was determined by plating on LB agar plates containing 50 mg/L ampicillin (Sigma).

**Nucleotide accession number**

The GenBank accession number for blaCMY-7 and associated upstream sequences is AY324388.

**Results**

**Search for ampR**

A disc induction assay was used to phenotypically determine whether blaCMY-7 was inducible. No induction was observed using this technique which suggested the absence of a functional AmpR. To confirm the absence of ampR, an upstream region of 524 base pairs was generated and sequenced (Figure 1). No ampR sequence was found. However, analysis of DNA sequences showed that 32 of the 38 bases
ampC expression and biological cost in *Salmonella*

of the AmpR binding region were present, suggesting an incomplete AmpR binding site.

**Special features of CMY-7 upstream sequences**

Analysis of upstream sequences of *bla*CMY-7 revealed a partial sequence of an insertion element, IS*Ecp1* (nucleotides 291–407; Figure 1). The start site of transcription for *bla*CMY-7 expression was mapped to nucleotide 341 (183 nucleotides upstream from the initiation codon), within the IS*Ecp1* sequence. This location suggested that *bla*CMY-7 expression was driven by putative −35 and −10 promoter elements located within this IS element (Figures 1 and 2). Interestingly, the −10 (TACAAT) and −35 (TTGAAA) elements located within IS*Ecp1* had more similarity (83%, 10/12 bases) to consensus *E. coli* −10 (TATAAT) and −35 (TTGACA) elements than the chromosomal *C. freundii* ampC promoter elements (58%, 7/12 bases) (Figure 1). Sequences further upstream of IS*Ecp1* (nucleotides 33–290) showed similarity to the *traA* gene sequence of IncI1 plasmid R64.25

**Expression of blaCMY-7**

Plasmid profile and relative copy number analysis revealed that pCMY-7 was present in two copies in *S. typhimurium* strain 100 (data not shown). The ampD of *S. typhimurium* strain 100 was used as a single copy gene reference for gene copy determination of *bla*CMY-7. *bla*CMY-7 is considered to have originated from the chromosomal ampC of *C. freundii* and a derepressed mutant is considered to produce high levels of AmpC conferring clinical resistance.3,18 Therefore, the level of *bla*CMY-7 expression in *S. typhimurium* strain 100 was compared with the expression of the chromosomal ampC of *C. freundii* strain 21 and *C. freundii* strain 21 M, a derepressed mutant, by primer extension analysis. The expression of the *bla*CMY-7 transcript in *S. typhimurium* strain 100 was ~965-fold higher than *C. freundii* strain 21 and ~4.1-fold higher than *C. freundii* 21 M. The expression data were normalized using 16S rRNA from the same RNA preparations.

**Effect of AmpC expression on growth and virulence**

An AmpC β-lactamase has been implicated to decrease growth and virulence in an *S. typhimurium* isolate which expressed the enzyme at high levels.16 To test this hypothesis, a *Salmonella* transconjugant (LT2::pCMY-7) which expressed *bla*CMY-7 from pCMY-7 and a transformant (LT2::pAH1) expressing a cloned *bla*CMY-7 were evaluated. The cefoxitin MIC values for the clinical isolate *S. typhimurium* 100, and strains LT2::pCMY-7 and LT2::pAH1 were ≥256 mg/L. The cefoxitin MIC was 2 mg/L for the recipient strain, LT2. The growth of *S. typhimurium* LT2 and LT2::pCMY-7 as determined by optical density at 600 nm and viable counts (Figure 3a and b). At 10 h post-inoculation, a 6–7 log increase in cfu/mL was noted for all three strains. However, the growth of LT::pAH1 was dramatically reduced (cfu/mL ~10⁶) compared to the other three *Salmonella* strains (cfu/mL ~10¹⁰) (Figure 3b).

The effect of high-level *bla*CMY-7 expression on the ability of *Salmonella* strains to invade mammalian cells was studied using gastric carcinoma cells. Approximately 8.3% of the *S. typhimurium* strain...
S. typhimurium to the invasion potential of 100 inoculum invaded the mammalian cells as revealed by invasion assays. Figure 4 demonstrates the invasion potential of the strains LT2, LT2:pCMY-7 and LT2:pAH1. The invasion potential is defined as the percentage of bacterial inoculum which invaded the cells. The strains were compared by setting the invasion potential of S. typhimurium expressing a plasmid-encoded AmpC β-lactamase, CMY-7. The data presented in this paper show that high-level expression of a plasmid-encoded ampC β-lactamase gene did not compromise the virulent phenotype of a clinical S. typhimurium strain. Biological cost of high-level ampC expression was observed for the transformant as demonstrated by the differences observed in the growth and invasion potential of the transconjugant compared to the transformant expressing bla<sub>CMY-7</sub>. Therefore, the biological cost of high-level AmpC production is compensated by plasmid-encoded factors and not controlled by repressing ampC expression.

The model system examining biological cost of ampC expression suggested that isolates of S. typhimurium required the co-acquisition of ampR leading to AmpR production thus keeping ampC expression at low levels. At the time of development of the Morosini model, only the inducible plasmid-encoded ampC gene, bla<sub>CMY-7</sub>, had been found in Salmonella. Therefore one of the objectives of this study was to determine how bla<sub>CMY-7</sub> was regulated. In the clinical isolate, S. typhimurium strain 100, induction could not be demonstrated and sequence analysis showed no ampR associated with bla<sub>CMY-7</sub>. However, only part of the AmpR binding site was present. Some plasmid-encoded ampC genes, such as bla<sub>CMY-2</sub> (bla<sub>CMY-2</sub>)<sup>1</sup> and bla<sub>CMY-3</sub> also have partial AmpR binding sites indicating putative chromosomal origins of these genes.<sup>26,29</sup>

Figure 3. (a) Representative growth curves of S. typhimurium strain 100, LT2, LT2:pCMY-7 and LT2:pAH1. Growth was monitored spectrophotometrically by measuring optical density (OD) at 600 nm. (b) Viable counts of bacteria at different time points of growth. The standard deviations were less than 11%.

![Graph](https://example.com/graph.png)

**Figure 4.** Invasion potential of S. typhimurium strain 100, LT2, LT2:pCMY-7 and LT2:pAH1. The invasion potential is defined as the percentage of bacterial inoculum which invaded the cells. The strains were compared by setting the invasion potential of S. typhimurium strain 100 at 100%. The data represent the results of two independent experiments done in triplicate. Error bars represent standard deviations.

The acquisition of antibiotic resistance in bacteria has been associated with certain types of biological costs for the organism. However, compensatory mutations within the organism can ameliorate biological cost without the loss of antibiotic resistance.<sup>12,26,27</sup> The biological consequences of high-level ampC expression in S. typhimurium has been examined by Morosini et al.<sup>10</sup> using a laboratory constructed model. Those data correlated reduced growth and a decreased capacity to invade mammalian cells with high-level ampC expression. The work presented here sought to take these observations one step further and examine the biological cost associated with a clinical isolate of S. typhimurium expressing a plasmid-encoded AmpC β-lactamase, CMY-7. The data presented in this paper show that high-level expression of a plasmid-encoded ampC β-lactamase gene did not compromise the virulent phenotype of a clinical S. typhimurium strain. Biological cost of high-level ampC expression was observed for the transformant as demonstrated by the differences observed in the growth and invasion potential of the transconjugant compared to the transformant expressing bla<sub>CMY-7</sub>. Therefore, the biological cost of high-level AmpC production is compensated by plasmid-encoded factors and not controlled by repressing ampC expression.

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ampC expression and biological cost in Salmonella

100 suggesting that plasmid copy number played a minimal role in overall expression of \( \text{bla}_{\text{CMY-7}} \).

High-level ampC expression in the clinical isolate \( S. typhimurium \) strain 100 did not correlate with a decrease in invasion potential or growth rate compared to LT2 or LT2:pCMY-7. The invasion potential (8.3%) observed for the \( S. typhimurium \) strain 100 was comparable to those reported for other \( S. enterica \) isolates (12.3%.16 14.5%).5 However, invasion potential can vary with the strain of \( S. enterica \) and type of mammalian cells used.16,35 The ~3-fold increase in invasion potential of LT2:pCMY-7 in comparison to LT2 was an interesting observation. Invasion of mammalian cells by \( S. enterica \) is a complex process that involves coordinated function of a number of gene products. Most of the invasion genes are clustered together in a region of the chromosome described as \( S. enterica \) pathogenicity island 1. It has been observed that certain genes present on a high molecular weight plasmid can also play an important role.36,37 The increase in invasion potential of the transconjugant LT2:pCMY-7 in comparison to LT2 suggests that the large plasmid which was transferred may encode invasion associated gene(s) in addition to \( \text{bla}_{\text{CMY-7}} \). A lower growth rate and invasion potential was observed for the transfectant, LT2:pAH1. These data support the observation by Morosini et al.16 that high-level ampC expression can affect the invasion and growth rate for \( S. enterica \). However, taken together, these data indicate that high-level production of AmpC \( \beta \)-lactamases in clinical isolates of \( S. enterica \) can be compensated in the organisms by mechanisms other than the repression of ampC expression.

\( S. enterica \) is a self-limiting disease and antibiotics are usually not prescribed to treat the infection.38 However, the incidence of multidrug-resistant \( S. typhimurium \) is on the rise39,40 and \( S. enterica \) infections may be a cause for concern especially in children and individuals with compromised immune function.38,41 Therefore, when a virulent pathogen such as \( S. enterica \) becomes multidrug-resistant, it poses a greater therapeutic challenge. The ability of \( S. typhimurium \) to maintain virulence in the presence of high-level ampC expression demonstrates the versatility of this virulent pathogen and the need for vigilance surveillance of these types of resistance mechanisms in \( S. enterica \) spp.

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References


