A Fitness Cost Associated with the Antibiotic Resistance Enzyme SME-1 β-lactamase

David C. Marciano, Omid Y. Karkouti and Timothy Palzkill

Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX 77030

1Corresponding author. Mailing address: One Baylor Plaza, Houston, TX 77030. Phone: 713-798-5601. Fax: 713-798-6802. Email: timothyp@bcm.edu
A FITNESS COST ASSOCIATED WITH SME-1 β-LACTAMASE

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Corresponding author: Timothy Palzkill.
Mailing address: One Baylor Plaza, Houston, TX 77030.
Phone: 713-798-5601. Fax: 713-798-6802. Email: timothyp@bcm.edu
ABSTRACT

The \textit{bla}_{TEM-1} \(\beta\)-lactamase gene has become widespread due to the selective pressure of \(\beta\)-lactam use and its stable maintenance on transferable DNA elements. In contrast, \textit{bla}_{SME-1} is rarely isolated and is confined to the chromosome of carbapenem resistant \textit{Serratia marcescens} strains. Dissemination of \textit{bla}_{SME-1} via transfer to a mobile DNA element could hinder the use of carbapenems. In this study, \textit{bla}_{SME-1} was determined to impart a fitness cost upon \textit{E. coli} in multiple genetic contexts and assays. Genetic screens and designed SME-1 mutants were utilized to identify the source of this fitness cost. These experiments established that the SME-1 protein was required for the fitness cost but also that the enzyme activity of SME-1 was not associated with the fitness cost. The genetic screens suggested that the SME-1 signal sequence was involved in the fitness cost. Consistent with these findings, exchange of the SME-1 signal sequence for the TEM-1 signal sequence alleviated the fitness cost while replacing the TEM-1 signal sequence with the SME-1 signal sequence imparted a fitness cost to TEM-1 \(\beta\)-lactamase. Taken together, these results suggest that fitness costs associated with some \(\beta\)-lactamases may limit their dissemination.
Horizontal gene transfer must overcome several barriers before stable maintenance of the transferred gene in the recipient bacteria can occur. DNA entry, avoidance of restriction systems and incorporation into the host replication machinery are necessary steps for transfer (THOMAS AND NIELSEN 2005). Although fulfilling these criteria permits a gene to transfer, the gene must also confer a selective advantage in order to expand within the bacterial population (BERG AND KURLAND 2002). Such is the case with the rapid dissemination of β-lactamase genes in response to the selective pressure of β-lactam use. A better understanding of the barriers to horizontal gene transfer and possible costs associated with maintenance of the transferred gene may facilitate the development of strategies to reduce the spread of antibiotic resistance.

β-lactamases provide bacterial resistance to β-lactam antibiotics by catalyzing the hydrolysis of these drugs. The widespread dissemination of β-lactamase genes represents an ongoing challenge to the efficacy of treatment with β-lactam antibiotics. A large collection of β-lactamases exists and can be classified into four groups (A-D) based on primary amino acid sequence homologies (AMBLER et al. 1991). The most prevalent plasmid borne β-lactamase in Enterobacteriaceae is the class A TEM-1 β-lactamase (SHAH et al. 2004). The spread of this β-lactamase to over 25 species of gram-negative bacteria has contributed to increased bacterial resistance to penicillins and early cephalosporins and thereby has reduced their efficacy as anti-bacterial agents. In response to increased bacterial resistance to these β-lactams, extended spectrum cephalosporins and β-lactamase inhibitors were introduced in the 1980’s. The selective pressure resulting from the introduction of these antibiotics has resulted in the rapid
evolution of TEM-1 β-lactamase. There are now greater than one hundred TEM-1 variants containing amino acid substitutions that allow the enzyme to hydrolyze extended-spectrum β-lactams and/or avoid inactivation by the β-lactamase inhibitors (www.lahey.org). To date, no TEM-1 variants have surfaced capable of conferring resistance to a third class of β-lactams, the carbapenems.

Carbapenem resistance is often associated with decreased cell wall permeability and the presence of class B or D β-lactamases that are distantly related to the class A β-lactamase TEM-1 (NAVON-VENEZIA et al. 2005). A few class A β-lactamases capable of hydrolyzing carbapenems (referred to as carbapenemases) have been discovered in clinical Enterobacteriaceae isolates. One such carbapenemase, SME-1, has been identified in several Serratia marcescens strains and is encoded by the chromosomal blaSME-1 gene (NAAS et al. 1994; YANG et al. 1990). Genomic analysis of several carbapenem resistant S. marcescens strains isolated from diverse geographical locations reveals a closer genetic relatedness among strains containing blaSME-1 than S. marcescens strains lacking the blaSME-1 gene and therefore they may represent a globally disseminated subtype of this species (QUEENAN et al. 2000). The blaSME-1 gene has not been identified on plasmids or other mobile genetic elements which may have restricted the spread of this gene.

Class A β-lactamases have no known function besides β-lactam hydrolysis and therefore incorporation of β-lactamase genes into a bacterial cell is not expected to alter metabolism (JAIN et al. 1999). It has been hypothesized, however, that class C β-lactamases could participate in recycling of cell wall components due to the structural similarity between β-lactam antibiotics and the DD-peptide bond found in the
peptidoglycan layer (BISHOP and WEINER 1992). A fitness cost associated with expression of the class C β-lactamase AmpC from Enterobacter cloacae in Salmonella enterica serotype Typhimurium has been documented (MOROSINI et al. 2000). However, no significant changes in the muropeptide composition of the peptidoglycan could be discerned. Regardless of the cause, the association with a fitness cost may explain why AmpC producing Salmonella are seldom isolated (MOROSINI et al. 2000).

Subsequent work by Hoissan et al, found that another class C β-lactamase, CMY-7, impaired Salmonella typhimurium strain LT2 growth and cell invasion when encoded on a high copy number plasmid (HOSSAIN et al. 2004).

In the course of working with the class A SME-1 carbapenemase, it was noted that cell lysis and occasional plasmid rearrangement events occurred in overnight cultures of Escherichia coli transformed with a plasmid encoding SME-1. These observations suggested a fitness cost may be associated with blaSME-1 in E. coli. The purpose of this investigation was to quantitatively establish the presence of a blaSME-1 associated fitness cost and then to determine the origins of the fitness cost. Genetic screens as well as designed mutant constructs were used to investigate specific features of the SME-1 gene as potential sources of the fitness cost. These methods revealed the requirement of the SME-1 enzyme in conferring a fitness cost to E. coli but suggest that enzymatic activity itself does not play a role.

**MATERIALS AND METHODS**

**Bacterial strains and cloning.** Unless otherwise noted, all enzymes were obtained from New England Biolabs (Ipswich, MA). E. coli K12 XL1-Blue strain (recA1 endA1
gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB lacI*ZAM15 Tn10 (Tet')]) was obtained from Stratagene (La Jolla, CA). *E. coli* B Ara⁺ and Ara⁻ strains were a gift from Richard E. Lenski (BOUMA and LENSKI 1988). pTP123 is a derivative of the pTrc99A vector encoding the LacI repressor and a multiple cloning site downstream of the P_{trc} promoter (PETROSINO et al. 1999). pTP123-TEM-1 and pTP123-SME-1 were constructed by inserting the *bla*<sub>TEM-1</sub> and *bla*<sub>SME-1</sub> genes, respectively, into the multiple cloning site of pTP123 (MAJIDUDDIN and PALZKILL 2003a; MAJIDUDDIN and PALZKILL 2003b). pUC18-Kn was constructed by releasing the kanamycin resistance gene from the pKRP11 plasmid (Reece and Phillips, 1995) with the *Hinc*II restriction enzyme and inserting the fragment into the end-filled pUC18 plasmid cut with *AatI/BsaI*. This resulted in a majority of the *bla*<sub>TEM-1</sub> gene being replaced by the kanamycin resistance gene. pUC18-Kn-TEM-1 and pUC18-Kn-SME-1 were constructed by inserting the *bla*<sub>TEM-1</sub> and *bla*<sub>SME-1</sub> genes from pTP123-TEM-1 and pTP123-SME-1, respectively, into the multiple cloning site of pUC18-Kn using the *SacI/XbaI* and *SacI/BamHI* restriction enzymes, respectively. The SME-1 truncation mutant (pTP123-Trunc.SME-1) is a fortuitously isolated spontaneous mutant of pTP123-SME-1 which possess a single base pair deletion of t363 (numbering according to NAAS *et al.* 1994) resulting in an amber STOP codon at amino acid position 81 (numbering according to AMBLER *et al.* 1991). The SME-1 ΔSTART mutant (pDCM104) was created by using Stratagene’s (La Jolla, CA) QuickChange kit with pTP123-SME-1 as template and the SME-STARTdel primer which mutates the first and second codons to create a SpeI site. All primers used in this study are listed in Table 1. The pTP123-SME-1 C69V:C238V construct was created via use of the QuickChange kit and primers SME-C69V-Top and SME-C238V-Top
simultaneously. Screening for positive clones was facilitated by the introduction of silent mutations creating a HindIII site by the SME-C69V-Top primer and both the creation of a SfoI site and destruction of a naturally occurring NdeI site by the SME-C238V-Top primer. The pTP123-SME-1 S70A:E166Q mutant was created step-wise using overlap PCR (HO et al. 1989). First, the E166Q SME-1 mutant was created using internal primers SME-E166Q-Top and SME-E166Q-Bot and external primers SME-Sac and SME-BamHI with pTP123-SME-1 (MAJIDUDDIN and PALZKILL 2003a) as template. In the first step, each half of the wild type SME-1 gene was amplified separately using one internal and one external primer. This incorporated the desired mutation into the complementary ends of the two products which were combined in a final PCR reaction also containing external primers. The full length product with the desired mutation was then digested with the appropriate restriction enzymes along with DpnI to remove residual, host-derived plasmid DNA and ligated into the target plasmid treated with calf intestinal phosphatase (CIP). The S70A:E166Q SME-1 double mutant was created by overlap PCR using the internal primers SME-S70A-Top and SME-S70A-Bot and external primers SME-Sac and SME-BamHI with pTP123-SME-1 E166Q as template. To facilitate the creation of the TEM(SS)SME construct (pDCM102), a modified version of pTP123-TEM-1 was generated that contained a silent AgeI site in the signal sequence coding region of blaTEM-1. This modified pTP123-TEM-1, named pTP251, was created by overlap PCR using internal primers TEM-AgeI-Top and TEM-AgeI-Bot and external primers PDb1a1 (PETROSINO et al. 1999) and PDb1a2. The pTP251 plasmid allows insertion of mature coding sequences behind the canonical blaTEM-1 signal sequence coding region. The SME-1 mature coding region was amplified by Pfu polymerase
(Stratagene, La Jolla, CA) using the primers TEM-SME and SME-BamHI and digested
with Agel/BamHI for ligation into similarly digested pTP251. To create the
SME(SS)TEM construct (pDCM106-8), an overlap PCR reaction combining three
products was used. Overlap PCR was used to create the following: product A was
derived from pTP123-TEM-1 using primers PDb1a and Pbla-ssSME-Bot, product B was
derived from pTP123-SME-1 using primers Pbla-ssSME-Top and ssSME-mTEM-Bot,
and product C was derived from pTP123-TEM-1 using primers ssSME-mTEM-Top and
PDb2a. Full length SME(SS)TEM insert was recovered by combining products A, B,
and C with external primers PDb1a and PDb2a, digested with SacI/XbaI and ligated into
similarly digested pTP123. The resulting SME(SS)TEM construct, pDCM106-8, has the
5’ UTR and ribosome binding site of blaTEM-1 followed by the encoded signal sequence of
blaSME-1 fused to the mature blaTEM-1 sequence. All genes and their hybrids cloned into
the pTP123 vector are under the transcriptional control of the Ptrc promoter. Isopropyl β-
D-1-thiogalactopyranoside (IPTG) was not used in the experiments because basal
expression levels from the Ptrc promoter were sufficient to confer ampicillin resistance to
all constructs expected to produce a functional β-lactamase. All PCR reactions utilized
Pfu polymerase obtained from Stratagene (La Jolla, CA). All primers were obtained
from Integrated DNA Technologies (Coralville, IA). Silent mutations resulting in the
introduction or deletion of restriction enzyme sites were engineered into primers by using
the Primer Generator program found at http://www.med.jhu.edu/medcenter/ (TURCHIN
and LAWLER 1999). All constructs and several clones isolated from the genetic screens
(see below) were sequenced using Applied Biosystems Instruments (Foster City, CA)
Prism Big Dye DNA sequencing with an ABI 3100 automated sequencer. Prior to use in
each experiment, each construct was transformed into *E. coli* K12 XL1-Blue or *E. coli* B
Ara$^+$/Ara$^-$ cells and spread on LB plates (Difco Laboratories, subsidiary of Becton,
Dickinson and Company, Sparks, MD) containing the appropriate selective antibiotic.
When possible, ampicillin was also included in LB plates to maintain functional (e.g.
SME-1 expressing) clones.

*E. coli* strains containing chromosomally encoded $bla_{TEM-1}$ (TP112) or $bla_{SME-1}$
(DCM105) inserted into the *pyrF* locus were created using lambda Red homologous
recombination in *E. coli* SW102 (WARMING et al. 2005). In generating *E. coli* TP112,
the $bla_{TEM-1}$ gene along with its native promoter was PCR amplified from pBG66
(HUANG et al. 1996) using primers *pyrF*-bla-top and *pyrF*-bla-bot to yield a PCR
product with ~33 nucleotides on each end capable of homologous recombination with the
*pyrF* locus. Transformation of *E. coli* SW102 with this PCR product resulted in the
deletion of codons F70 to R131 of *pyrF* and insertion of $bla_{TEM-1}$. Insertion of $bla_{SME-1}$
into the *pyrF* locus was initiated by creating a plasmid, pDCM105, that encodes $bla_{SME-1}$
downstream of the 5’ UTR of $bla_{TEM-1}$. The plasmid pDCM105 was generated by an
overlap PCR reaction using pTP123-TEM-1 template with primers PDBla1 and Pbla-
ssSME-bot combined with a PCR fragment from pTP123-SME-1 template using primers
Pbla-ssSME-top and SME-BamHI. Amplification of $bla_{SME-1}$ from pDCM105 using the
primers PDBla1 and SME-1STOP-pyrF384bot yielded a PCR product with 146
nucleotides of homology with the 5’ UTR of $bla_{TEM-1}$ chromosomally encoded in TP112
and 26 nucleotides of homology with a region of *pyrF* downstream of the $bla_{TEM-1}$
insertion. Transformation of *E. coli* TP112 with the pDCM105 derived PCR product
replaced $bla_{TEM-1}$ with $bla_{SME-1}$ and further deleted *pyrF* codons E131 to V147, thus
creating strain DCM105 with $bla_{SME-1}$ under the control of $P_{bla}$. P1 transduction was subsequently utilized to move the chromosomally encoded $bla_{TEM-1}$ gene of *E. coli* TP112 into the *E. coli* B Ara$^+$ /Ara$^-$ strains (DCM106 and DCM107, respectively). Similarly, P1 transductants derived from DCM105 were utilized to move $bla_{SME-1}$ into the *E. coli* B Ara$^+$ /Ara$^-$ strains (DCM108 and DCM109, respectively). Due to the deficiency of *recA* in the parental SW102 strain, the low-copy number plasmid pGE591 supplied the RecA protein in *trans* to enhance P1 phage production in the donor strains, *E. coli* TP112 and DCM105 (WEISEMANN and WEINSTOCK 1988). The P1 transduction protocol is based upon a protocol found in “A short course in microbial genetics” (MILLER 1992).

**Genetic screen for spontaneous suppressors of a SME-1 mediated fitness cost.**

Spontaneous suppressors of the SME-1 mediated fitness cost were selected by passaging *E. coli* B Ara$^+$ containing pTP123-SME-1 for several days in LB media. Individual clones of pTP123-TEM-1 or pTP123-SME-1 were inoculated for growth overnight with shaking at 37°. Every 12 hours, 100 µl of culture was transferred to 10 ml of fresh LB broth containing chloramphenicol and grown again with shaking at 37°. Individual clones with a growth advantage are expected to overtake the culture. After 48 hours, a 1:10$^6$ dilution of the passaged culture was spread on LB plates containing 12.5 µg/ml chloramphenicol to obtain isolated colonies. DNA was isolated from seven pTP123-SME-1 clones and four pTP123-TEM-1 clones and subjected to restriction digest analysis. These clones were also patched onto LB plates containing 12.5 µg/ml chloramphenicol and plates containing both chloramphenicol and 100 µg/ml ampicillin to determine their resistance profile. As several of the pTP123-SME-1 clones possessed
aberrant restriction digests and/or sensitivity to ampicillin, five of these clones were sequenced resulting in four unique clones being isolated (Cp.1-4).

A similar selection for SME-1 suppressors was performed in LB broth containing both 12.5 µg/ml chloramphenicol and 100 µg/ml ampicillin. Again, cultures containing pTP123-TEM-1 or pTP123-SME-1 were allowed to grow 12 hours before being transferred into fresh LB media containing both chloramphenicol and ampicillin. At 96 hours, individual clones were obtained by spreading a 1:10^6 dilution of the passaged culture on LB plates containing chloramphenicol and 100 µg/ml ampicillin. Seven pTP123-SME-1 clones were sequenced resulting in three unique clones being isolated (CpAp.1, 2, 3).

**Library construction and selection.** Mutagenic PCR via inclusion of 0.0625 mM MnCl$_2$ was performed with 5U Taq polymerase (Promega, Madison, WI), 0.2 mM dNTPs (Bioline, Randolph, MA), 1.5 mM MgCl$_2$, 1X Taq polymerase buffer (Promega, Madison, WI), 100 ng of each primer SME-Sac and SME-BamHI and 45 ng of pTP123-SME-1 template in a 100 µl reaction. A second series of 100 µl mutagenic PCR reactions were performed utilizing either 2.5 or 0.25 ng of the first reaction’s product as template with either 0.0625 or 0.15 mM MnCl$_2$ (a total of four reactions performed in duplicate). The PCR reaction parameters were as follows: 95 °C for 1 min; followed by 30 cycles of 95 °C for 1 min, 50 °C for 2 min, and 72 °C for 3 min; ending with 72 °C for 10 min. All 8 mutagenic PCR reactions were pooled and column purified using a Qiagen PCR purification kit (Qiagen Inc., Valencia, CA) before sequential digestion with SacI/BamHI/DpnI. Insert DNA was gel purified and ligated into CIP treated pTP123
digested with *SacI/BamHI*. The ligation was phenol/chloroform extracted, transformed into electrocompetent *E. coli* K12 XL1-Blue and spread onto LB plates containing chloramphenicol. A total of $1.72 \times 10^5$ cfu’s were pooled. Plasmid DNA was isolated from two clones and sequencing showed six nucleotide changes in one clone and two nucleotide changes in the other. The library was subjected to two independent selections by passaging in LB broth with 12.5 µg/ml chloramphenicol or in LB broth with 12.5 µg/ml chloramphenicol and 100 µg/ml ampicillin as described above for the selection of spontaneous mutants. At 96 hours, DNA was isolated from individual clones and sequenced.

**Growth curves.** Growth curve data was obtained by measuring cell density at OD$_{600}$ on a Beckman-Coulter (Fullerton, CA) DU 800 Spectrophotometer. Isolated clones of each construct, in either *E. coli* K12 XL1-Blue or *E. coli* B Ara$^+$, were inoculated into 2 ml of LB broth with chloramphenicol and incubated overnight. Cultures were then diluted to an OD$_{600}$ of 0.1 in 10 ml of LB with chloramphenicol and allowed to shake vigorously (295 rpm) at 37°C. OD$_{600}$ was monitored over a time course of 23 hours. The data represents an average of at least three independent experiments.

**Culture viability.** Individual clones of *E. coli* K12 XL1-Blue transformed with pTP123, pTP123-TEM-1, pTP123-SME-1, pTP123-SME-1 S70A:E166Q, pDCM102, pDCM106-8, pUC18-Kn, pUC18-Kn-TEM-1 or pUC18-Kn-SME-1 were inoculated into 10 ml LB broth containing either chloramphenicol or kanamycin (according to plasmid resistance marker) and allowed to grow 24 hours with vigorous shaking (295 rpm) at 37°C. Overnight cultures were diluted 1:10$^6$ and 100 µl was spread in duplicate onto LB plates
and allowed to grow overnight at 37°. The number of colony forming units per ml was
determined for each culture and expressed relative to the number of colony forming units
per ml obtained from an overnight culture containing empty vector (pTP123 or pUC18-
Kn). The data represents an average of at least six independent experiments. The $t$-test
was utilized to assign statistical significance by comparison against data obtained with
the relevant empty vector.

**Competition experiments.** In order to directly compare fitness between two strains in a
mixed culture, *E. coli* B strains differing by the ability to utilize arabinose (Ara$^+$ and Ara$^-$
) were transformed with the desired plasmids and allowed to compete during overnight
growth. Overnight starter cultures of the competing clones were mixed in a 50:50 ratio
and 200 µl of the mixed culture was used to inoculate 20 ml of LB broth containing
chloramphenicol. During the course of the experiment, dilutions of the mixed culture
were spread onto tetrazolium arabinose indicator (TA) plates to determine the
contribution of each strain to the total population. On TA plates, *E. coli* B Ara$^+$ appears
pinkish-white while the Ara$^-$ strain appears dark red (BOUMA and LENSKI 1988;
LEVIN *et al.* 1977; LENSKI 1988). Competitions were performed in both strain
contexts (i.e. Ara$^+$ pTP123-TEM-1 vs. Ara$^-$ pTP123-SME-1 and Ara$^+$ pTP123-TEM-1 vs.
Ara$^+$ pTP123-SME-1). Because no strain specific effects were observed with the plasmid
constructs, the competition data was averaged between the strain contexts. The
competitions of plasmid encoded $\beta$-lactamase genes were followed overnight. For strains
possessing chromosomally encoded $\beta$-lactamase genes, competitions were conducted
over 3 days during which time 200 µl of the culture propagated into 20 ml of fresh LB
media every 16 hours. Data obtained from these experiments represents an average of at least four independent experiments and was used to calculate a selection rate constant for each competition. The selection rate constant is the difference of the realized Malthusian parameters associated with the competing *E. coli* B strains, and is dependent upon the relative growth rates of the competing strains (LENSKI *et al.* 1991). It was determined by regression analysis using SigmaPlot with the following equation: \( \log e R(t) = \log e R(0) + st \), where \( R \) is the ratio of the prevalence of the competing strains, \( t \) is time in days and \( s \) is the selection rate constant having units of day\(^{-1}\) (LENSKI *et al.* 1994; TRAVISANO and LENSKI 1996).

**Minimum inhibitory concentration (MIC) determination.** \( \beta \)-lactam resistance levels were utilized to determine if the chimeric TEM(SS)SME (pDCM102) and SME(SS)TEM (pDCM106-8) clones conferred functional *in vivo* resistance levels equivalent to pTP123-SME-1 and pTP123-TEM-1, respectively. Both E-test strips (AB BIODISK, Piscatway, NJ) and broth dilution methods were utilized. E-test strips containing the \( \beta \)-lactams ampicillin or amoxicillin + clavulanic acid were placed upon LB plates spread with a one tenth dilution of an overnight culture of *E. coli* B transformed with either pTP123-TEM-1, pTP123-SME-1, pDCM102 or pDCM106-8. For broth dilution MIC, 2-fold dilutions of ampicillin were tested in a 96-well format in which each well with contained \( 1 \times 10^4 \) bacteria in 100 \( \mu l \). The 96-well plate was sealed and allowed to shake over night at 37\(^{\circ}C\) before scoring for growth. Each assay was conducted in triplicate using independent overnight cultures. The generally accepted level of significance of \( \pm 1 \) two-fold dilution
was employed determine if the chimeras displayed a significant effect on *in vivo* activity relative to the relevant wild type construct.

**Plasmid loss from strains grown in LB media.** The effect of wild type and mutant SME-1 genes on plasmid stability in *E. coli* B was determined by monitoring the rate of plasmid loss over time. Isolated colonies were inoculated into 10 ml of LB media lacking a selective antibiotic and grown at 37 °C with vigorous shaking for 24 hours. Every 24 hours, 5 µl of culture was transferred to 10 ml of fresh LB. Additionally, every 24 hours, dilutions were spread in duplicate onto LB plates and LB plates containing a selective antibiotic (either chloramphenicol for the pTP123 series or kanamycin for the pUC18-Kn series). Because plasmid loss was evident in the pUC18-Kn-SME-1 construct after just 24 hours of growth, the possibility of plasmid loss on the original LB agar plate was investigated by resuspending colonies in LB broth, diluting and directly spreading onto LB plates and LB plates containing kanamycin. Colonies were counted to determine the percentage of bacteria that retained plasmid as ascertained by the ability to grow on plates containing antibiotic. Experiments were conducted at least in duplicate.

**RESULTS**

**Establishing the existence of a bla<sub>SME-1</sub> mediated fitness cost in *Escherichia coli*.** The observance of plasmid instability and cell lysis in overnight cultures containing plasmids encoding bla<sub>SME-1</sub> suggested the possibility of a fitness cost associated with the SME-1 gene in *Escherichia coli*. In order to quantify these observations, several independent assays were utilized in multiple genetic contexts to delineate a SME-1 mediated fitness cost in *E. coli*. Initially, the effect of the SME-1 gene on the growth of *E. coli* B and K12
XL1-Blue strain was determined by measuring the optical density of the culture over time. As seen in Figure 1A, the presence of \textit{bla}_{\text{TEM-1}} encoded on the pTP123 plasmid (pTP123-TEM-1) had little impact on the growth rate of \textit{E. coli} K12 XL1-Blue relative to strains transformed with empty vector (pTP123). However, \textit{E. coli} K12 XL1-Blue transformed with plasmid expressing SME-1 β-lactamase (pTP123-SME-1) exhibited an extended lag phase and an overall reduction of the final cell density in stationary phase. Similar results were obtained in \textit{E. coli} B cells (data not shown).

Culture viability experiments were utilized to determine the effect of the \textit{bla}_{\text{SME-1}} gene on the number of colony forming cells present in overnight cultures of \textit{E. coli} K12 XL1-Blue. Overnight cultures of \textit{E. coli} K12 XL1-Blue transformed with plasmid encoding \textit{bla}_{\text{SME-1}} were diluted and spread onto LB plates. The number of colony forming units per ml was determined and is presented in Figure 2 relative to the number of colony forming units per ml obtained from an overnight culture containing empty vector. Relative to empty vector, the \textit{bla}_{\text{SME-1}} gene confers reduced culture viability when encoded by either a pTrc99A based vector (pTP123 series, P<0.001) or a pUC18 based vector (pUC18-Kn series, P<0.001) (Figure 2). The gene encoding TEM-1, \textit{bla}_{\text{TEM-1}}, had no statistically significant effect on the number of colony forming cells in either plasmid context as determined by this assay (pTP123-TEM-1, P=0.62; pUC18-Kn-TEM-1, P=0.07). These results are consistent with a \textit{bla}_{\text{SME-1}} mediated fitness cost that is present in multiple genetic contexts.

To quantitate the fitness cost associated with the SME-1 gene, competition experiments were performed (BOUMA and LENSKI 1988). Initially, Ara$^+$ or Ara$^-$ \textit{E. coli} B strains were transformed with either pTP123-SME-1 or empty vector pTP123,
mixed in a 50:50 ratio and competed over a 24 hour time course. The relative contribution of each construct to the total culture population was tracked by spreading dilutions onto tetrazolium-arabinose (TA) indicator plates. *E. coli B Ara*⁺ is capable of utilizing arabinose and appears white or light pink on TA indicator plates while an *E. coli B Ara*⁻ strain is unable to utilize arabinose and appears dark red on TA indicator plates (BOUMA and LENSKI 1988; LEVIN et al. 1977; LENSKI 1988). Within five hours, less than 10% of the mixed culture contained the strain harboring the pTP123-SME-1 plasmid. As a more stringent test of fitness, the Ara⁺ or Ara⁻ *E. coli B strain containing the pTP123-SME-1 plasmid was competed against the reciprocal Ara⁺/ Ara⁻ E. coli B strain containing isogenic pTP123-TEM-1. After an overnight competition, 98 ± 3% of the culture was composed of the pTP123-TEM-1 containing strain. To facilitate comparisons between experiments, the selection rate constant of each competition was determined (Table 3). The negative selection rate constant, -7.0 ± 2.4 day⁻¹, represents a significant fitness cost associated with carriage of the pTP123-SME-1 plasmid relative to pTP123-TEM-1. This provides direct evidence of a *bla*<sub>SME-1</sub> mediated fitness cost in *E. coli B*.

To determine if the fitness cost due to *bla*<sub>SME-1</sub> was dependent upon copy number, the *bla*<sub>SME-1</sub> was transferred to the chromosome of the *E. coli* Ara⁺/Ara⁻ strains and competitions against *E. coli* Ara⁺/Ara⁻ strains encoding a chromosomal *bla*<sub>TEM-1</sub> gene were performed. Although a strain dependent effect is apparent, in all competitions the *E. coli B strain encoding a chromosomal copy of *bla*<sub>TEM-1</sub> outcompeted the reciprocal strain encoding *bla*<sub>SME-1</sub> (Table 3). It should be noted that the chromosomally encoded *bla*<sub>SME-1</sub> differs from the vector encoded *bla*<sub>SME-1</sub> in that it possess the 5’ UTR of *bla*<sub>TEM-1</sub>. Although this establishes the presence of a fitness cost associated with the *bla*<sub>SME-1</sub>
coding region, the difference of selection coefficients observed between chromosomally encoded and plasmid encoded \( \text{bla}_{\text{SME-1}} \) could be copy number or 5’ UTR dependent. However, the data is consistent with the \( \text{bla}_{\text{SME-1}} \) gene conferring a fitness cost to \( E. \text{coli} \) B even when in a chromosomal context.

The persistence of antibiotic resistance genes in the absence of selection suggests a neutral fitness effect. A lack of a fitness cost may contribute to the failure of antibiotic cessation to remove reservoirs of \( \beta \)-lactam resistance in a natural environment (LIEBANA et al. 2006). In order to address the persistence of \( \text{bla}_{\text{SME-1}} \), the effect of the gene on the rate of plasmid loss in the absence of selection was ascertained. Cultures were inoculated with isolated colonies and then propagated into fresh LB media every 24 hours for 5 days. The proportion of the culture harboring plasmid was determined by comparing the number of colony forming units obtained on LB plates to the number of colony forming units obtained on plates with the appropriate selective antibiotic (chloramphenicol for the pTP123 series and kanamycin for the pUC18-Kn series). By day 3, a majority of viable cells in the culture had lost the pTP123-SME-1 plasmid (Figure 3). In contrast, neither the pTP123 nor the pTP123-TEM-1 vectors displayed any appreciable levels of plasmid loss during the course of the experiment. In the context of the pUC18-Kn vector, the \( \text{bla}_{\text{SME-1}} \) gene yielded a \( 57 \pm 22\% \) loss of the SME-1 construct within 24 hours while the pUC18-Kn-TEM-1 culture displayed no plasmid loss within 24 hours (data not shown). Resuspension and dilution of pUC18-Kn-SME-1 colonies directly taken from the agar plate showed no plasmid loss occurring on the plate itself (109 \( \pm \) 21\%). The simplest interpretation of these results is that plasmid free cells arise in the culture by passive loss of the SME-1 encoding plasmids and subsequently expand in
the population to overtake the culture. However, these data do not distinguish between plasmid segregation, plasmid stability or fitness cost as being the root cause of plasmid loss mediated by \textit{bla}_{SME-1} (LENSKI and BOUMA 1987). Taken together, the results of these experiments indicate the presence of the SME-1 gene results in reduced growth, lower culture viability, impaired fitness and plasmid loss in \textit{E. coli} and that these effects are not specific to a plasmid or strain context.

**Genetic analysis of the \textit{bla}_{SME-1} mediated fitness cost.** To determine the source of the \textit{bla}_{SME-1} mediated fitness cost, mutants of \textit{bla}_{SME-1} that ameliorate the fitness cost were isolated under several selection schemes. An overnight culture of \textit{E. coli} B containing the pTP123-SME-1 plasmid was passaged into fresh LB media with chloramphenicol for one week. Clones with mutations that confer a fitness advantage are expected to expand in number and dominate the culture. The presence of chloramphenicol in the culture maintains the chloramphenicol-resistant plasmid backbone but does not select for a functional SME-1 gene. As a control, \textit{E. coli} B containing pTP123-TEM-1 were passaged in parallel. To determine the effect of passaging on \(\beta\)-lactamase expression, each culture was diluted and spread onto plates containing the \(\beta\)-lactam ampicillin at the end of the experiment. Also, culture supernatant was immunoblotted with anti-\(\beta\)-lactamase sera to determine if \(\beta\)-lactamase was being produced and secreted into the media. As determined by these experiments, expression of TEM-1 \(\beta\)-lactamase was not affected by passaging. However, the SME-1 culture lost \(\beta\)-lactamase expression as determined by a lack of ampicillin resistant clones and immunoblotting results (data not shown). DNA sequencing of the SME-1 gene from passaged clones revealed that an IS1
transposon had been inserted into the SME-1 gene. This insertion event occurred at least three independent times (Table 2, Cp.2-4) as evident by differences between clones in the exact insertion point of the IS1 element (nucleotides 453, 457 and two at 460; numbering according to Nass et al. 1994). Also, one passaged pTP123-SME-1 clone was found to have an 11 base pair deletion in the N-terminus of the \textit{bla}_{SME-1} coding region which resulted in a frame shift and truncation at Phe19 (Table 2, Cp.1). Although such genetic events are expected to occur with low frequency in \textit{E. coli} cultures, the emergence of these mutants to replace the original wild type construct suggests these clones must have a selective advantage in the absence of a \(\beta\)-lactam antibiotic. No DNA rearrangements or mutations were detected in clones isolated from the pTP123-TEM-1 culture passaged in parallel under the same conditions. This data further supports the existence of a \textit{bla}_{SME-1} mediated fitness cost and provides evidence for the requirement of the SME-1 enzyme in conferring the fitness cost.

In a similar experiment, a strain containing the pTP123-SME-1 plasmid was passaged in LB media containing both chloramphenicol and 100 \(\mu\)g/ml ampicillin. Inclusion of ampicillin prevents the emergence of non-functional, insertion or deletion mutants in the population and thereby requires alternative means to suppress the \textit{bla}_{SME-1} mediated fitness cost. The pTP123-TEM-1 construct was passaged in parallel as a control. After 4 days of passaging, DNA was isolated and sequenced from individual clones. Again, clones from the pTP123-TEM-1 culture displayed the expected DNA migration pattern and did not have any mutations as determined by DNA sequencing. However, clones from the pTP123-SME-1 culture clones with aberrant DNA migration had either IS5 or IS10 elements inserted downstream of the \textit{bla}_{SME-1} gene (Table 2,
CpAp.2 and CpAp.3). IS elements mediating polar effects on gene transcription have been described and may be altering SME-1 protein levels in this case (SCHNEIDER and LENSKI 2004). In several clones with the expected DNA mobility, a nonsynonymous point mutation occurred causing a single N20Y amino acid substitution in the SME-1 signal sequence near the signal cleavage site.

In order to obtain more information concerning the source of the blaSME-1 associated fitness cost, a library of SME-1 mutant clones was created by error-prone PCR. As with the selection for spontaneous suppressors of the fitness cost, this library was subjected to selection by passaging either in the presence or absence of ampicillin for several days with the expectation that clones with a fitness advantage would expand in number and dominate the culture. Signal sequence mutations were present in all of the clones isolated from the library under both passaging conditions. In some cases, mutations outside of the signal sequence were found in combination with signal sequence mutations (Table 2, Library clones).

The prevalence of signal sequence mutations may reflect a direct role of the SME-1 signal sequence in the fitness effect or could indicate an indirect suppression of this phenomenon by preventing normal delivery of the SME-1 protein to the periplasmic space. In order to address this issue, periplasmic extracts were obtained from the most prevalent mutants, F19L and N20S, and were analyzed by immunoblot. As with wild type SME-1, both of these signal sequence mutants delivered >95% of the SME-1 protein to the soluble fraction of the periplasmic extract and did not have any discernable effect on expression levels (data not shown). The signal sequence substitutions F19L and N20S
therefore do not appear to suppress the fitness effect by preventing delivery of the mature SME-1 to the periplasmic space.

**Defining structural components of bla\textsubscript{SME-1} involved in imparting a fitness cost to *E. coli*.** The repeated isolation of non-functional SME-1 clones with deletions or insertions from the genetic screens suggests a requirement for the SME-1 protein in conferring a fitness effect. A frameshift mutant of bla\textsubscript{SME-1} (pTP123-Trunc.SME-1) was fortuitously isolated and found to exhibit a growth curve similar to *E. coli* K12 XL1-Blue transformed with empty vector pTP123 or pTP123-TEM-1 (Figure 1B). Overnight competition of the wild type pTP123-SME-1 construct versus the truncation mutant yielded a selection rate constant of -2.2 ± 1.3 day\(^{-1}\), indicating a competitive advantage conferred by the frameshift mutation (Table 3). This confirms that some component of the SME-1 protein is necessary to mediate the fitness cost associated with bla\textsubscript{SME-1}. To further confirm that the SME-1 protein was necessary to confer a fitness cost, the ATG START codon of the SME-1 gene was mutated to ACT for the purpose of preventing translation. Competition between *E. coli* B Ara\(^{+}\)/Ara\(^{-}\) strains transformed with either the wild type SME-1 construct or the START codon mutation construct (pTP123-SME\_START) resulted in a selection rate constant similar to that obtained from the competition of wild type SME-1 against the SME truncation mutant (Table 3). This again supports the hypothesis that the SME-1 protein is involved in the fitness cost. To further confirm the absence of a fitness cost associated with the pTP123-SME-1\_START mutant, a competition of *E. coli* B Ara\(^{+}\)/Ara\(^{-}\) strains containing either pTP123-SME-1\_START or the pTP123-TEM-1 construct was conducted. Competition of wild type TEM-1 against the START codon mutant of SME-1 resulted in a selection rate constant of 0.34 ± 0.71 day\(^{-1}\) (Table 3). This
data indicated no statistically significant difference in fitness between these constructs. The START codon mutant is therefore a suppressor of the SME-1 mediated fitness effect as it out-competes wild type pTP123-SME-1 and lacks any detectable fitness cost as determined by competition against pTP123-TEM-1. This confirms that at least some portion of the fitness cost is associated with an intrinsic characteristic of the SME-1 protein.

Although a fitness effect of the SME-1 protein related to translation, processing or export was not excluded by the data, we chose to investigate the role of several unique structural features of SME-1 in mediating a fitness cost. Specifically, the role of SME-1’s carbapenemase activity in conferring a growth defect upon *E. coli* was tested by creating a double mutant which removes residues critical for hydrolysis of β-lactam antibiotics. In agreement with studies of structurally related class A β-lactamases (CHEN et al. 2005a; CHEN et al. 2005b; MATAGNE and FRERE 1995; MEROUEH et al. 2005; MINASOV et al. 2002; NUKAGA et al. 2003; STRYNADKA et al. 1992), the S70A:E166Q mutant of SME-1 is unable to confer detectable resistance to the β-lactam ampicillin. Also, the disulfide bridge near the SME-1 active site (SOUGAKOFF et al., 2002) was tested for its role in conferring a fitness cost via a SME-1 double mutant, C69V:C238V. Neither the ablation of enzyme activity in pTP123-SME-1 S70A:E166Q nor the removal of the unique disulfide bridge in pTP123-SME-1 C69V:C238V alleviates the SME-1 mediated toxicity as determined by the effect of these mutants on *E. coli* K12 XL1-Blue growth curves (Figure 1B). *E. coli* K12 XL1-Blue transformed with the pTP123-SME-1 S70A:E166Q construct also exhibits decreased culture viability, similar to wild type SME-1, as measured by the number of colony forming cells per ml relative
to empty vector (Figure 2). A competition between *E. coli* B strains containing either the wild type pTP123-SME-1 construct or the catalytically deficient S70A:E166Q SME-1 mutant resulted in a stable population with neither construct predominating in the culture as indicated by a selection rate constant of -0.9 ± 1.4 (Table 3). The SME-1 disulfide bond mutant was also used in competition experiments against *E. coli* B containing wild type SME-1. The C69V:C238V double mutant did not suppress the SME-1 mediated fitness cost and may possibly exacerbate the fitness cost phenotype (Table 3). Although some component of the SME-1 protein is associated with a fitness cost, neither enzyme activity nor disulfide bridge formation are requisite for the fitness cost of the SME-1 β-lactamase in *E. coli*.

In light of the repeated isolation of signal sequence mutants when selecting for enhanced fitness of pTP123-SME-1 containing *E. coli*, a construct was created that replaced the SME-1 signal sequence with the TEM-1 signal sequence and is designated as TEM(SS)SME. As measured by the minimum inhibitory concentration of ampicillin and amoxicillin + clavulanic acid, the TEM(SS)SME and pTP123-SME-1 clones conferred similar levels of β-lactam resistance (ampicillin MIC = 512 μg/ml for each clone and amoxicillin + clavulanic acid MIC = 32 μg/ml and 8-12 μg/ml for pTP123-SME-1 and TEM(SS)SME, respectively). *E. coli* K12 XL1-Blue transformed with the TEM(SS)SME construct displays a growth curve similar to *E. coli* K12 XL1-Blue transformed with pTP123-TEM-1 (Figure 1C). Additionally, TEM(SS)SME expressing *E. coli* K12 XL1-Blue cells no longer display the reduced culture viability associated with expression of wild type SME-1 (Figure 2). The importance of the SME-1 signal sequence in conferring a fitness cost upon *E. coli* was further investigated by competing
strains containing the TEM(SS)SME construct against wild type SME-1 and wild type TEM-1 containing constructs, separately. As seen in Table 3, the *E. coli* B strain carrying pTP123-SME-1 suffers a significant competitive disadvantage relative to the strain containing the TEM(SS)SME plasmid. Also, in competition experiments of the TEM(SS)SME construct versus wild type TEM-1 there is little evidence of any residual fitness cost associated with the TEM(SS)SME construct (Table 3). The TEM(SS)SME chimera consistently abrogates the SME-1 mediated effects upon *E. coli* growth, viability and fitness.

The ability of the TEM-1 signal sequence to suppress the aforementioned effects of SME-1 may be due a direct role of the SME-1 signal sequence in the fitness cost. In order to determine if the SME-1 signal sequence was sufficient to confer a fitness cost, a construct was created by fusing the SME-1 signal sequence coding region to the mature coding region of the TEM-1 gene and is designated SME(SS)TEM. As seen with the TEM(SS)SME construct, the SME(SS)TEM construct affords equivalent levels of β-lactam resistance as seen in *E. coli* B transformed with pTP123-SME-1 (ampicillin MIC = >4096 µg/ml for each clone; amoxicillin + clavulanic acid MIC = 12 µg/ml for each clone). Although the growth curve of the SME(SS)TEM construct is similar to the pTP123-TEM-1 growth curve (Figure 1C), the resolution of this assay may not be sensitive enough to detect subtle differences in growth rates. Culture viability is reduced for *E. coli* K12 XL1-Blue transformed with the SME(SS)TEM compared to pTP123-TEM-1 (Figure 2). The reduction in culture viability, however, is not as pronounced as that observed with wild type pTP123-SME-1. Competition of the SME(SS)TEM construct against wild-type pTP123-TEM-1 shows that the presence of the
SME-1 signal sequence imparts a fitness cost to the blatem-1 gene. The selection rate constant of -1.4 ± 1.1 obtained from this competition is also lower than that obtained when competing full-length SME-1 against TEM-1 (Table 3). Additionally, competition of pTP123-SME-1 against the SME(SS)TEM construct results in the fusion construct overtaking the wild-type SME-1 construct. In this case, a selection rate constant of zero would be expected if the entirety of the SME-1 fitness cost lies within the SME-1 signal sequence coding region. This result may indicate that the SME-1 signal sequence requires the context of the SME-1 mature protein to exert its entire fitness effect. Nevertheless, although the SME(SS)TEM construct cannot conclusively account for the entirety of the blasme-1 mediated fitness cost, these results reveal a significant contribution of the SME-1 signal sequence in conferring a fitness cost to E. coli.

In order to confirm the results of competition experiments, spontaneous plasmid loss was also monitored for several of the SME-1 mutant constructs. In agreement with previous assays, no difference could be discerned between strains containing the pTP123-SME-1 construct and the isogenic catalytic knock-out, S70A:E166Q (Figure 3). Also consistent with previous data, replacement of the SME-1 signal sequence with the TEM-1 signal sequence (TEM(SS)SME) suppressed any fitness cost as evident by maintenance of this construct until the terminus of the experiment. Fusing the SME-1 signal sequence to the mature TEM-1 coding region (SME(SS)TEM) is sufficient to confer rapid plasmid loss at a rate similar to what is observed with full-length SME-1. Taken together, these results indicate that the SME-1 protein results in a negative fitness effect upon E. coli and that the SME-1 signal sequence contributes to this effect.
DISCUSSION

Antibiotic resistance that occurs via modification of an antibiotic target often results in a fitness cost to the bacteria under permissive conditions. This suggests that the removal of antibiotic pressure will reduce the prevalence of resistant bacteria. However, the effectiveness of this strategy is dependent upon a fitness cost that can be overcome or reduced in several ways (ANDERSSON 2006; BOUMA and LENSKI 1987; LENSKI 1998). First, antibiotic resistance genes are often genetically linked in the form of multi-resistant mobile DNA elements and selection of one resistant determinate can result in the maintenance of other resistance genes by linkage (BEAN et al. 2005; ENNE et al. 2001; WELDHAGEN 2004). Second, fitness costs are typically negated by the appearance of compensatory mutations which alleviate the fitness cost while preserving the resistance phenotype (BESIER et al. 2005; BJÖRKMAN et al. 1998; BJÖRKMAN et al. 2000; BOUMA and LENSKI 1987; KUGELBERG et al. 2005; LEVIN et al. 2000; LUO et al. 2005; NILSSON et al. 2006; SCHRAG et al. 1997; ZHANG et al. 2006). Without a significant fitness cost, there is no selective pressure to drive a loss of the resistance determinant. Finally, multiple routes of resistance can exist and be highly variable with regards to the fitness costs they engender (BJÖRKMAN et al. 1998; GAGNEUX et al. 2006; KASSEN and BATAILLON 2006). Therefore, a spectrum of resistant clones can exist; some with no fitness costs or even enhanced fitness under permissive conditions.

In the case of SME-1 β-lactamase expression in E. coli B and E. coli K12 XL1-Blue, the burden on the host bacterium is significant as determined by growth curves, culture viability, plasmid instability and competition against E. coli B expressing TEM-1
β-lactamase. The fitness cost of $bla_{SME-1}$ in $E. coli$ appears to be present when encoded on either a pTrc99A derived (pTP123) or a pUC18 derived (pUC18-Kn) plasmid as evident by a decrease in culture viability and an increase in the rate of plasmid loss relative to isogenic plasmids encoding $bla_{TEM-1}$. In using high-copy number plasmids, gene dosage effects may amplify the SME-1 mediated fitness costs observed. However, even when chromosomally encoded in the $E. coli$ Ara⁺/Ara⁻ strains, a $bla_{SME-1}$ mediated fitness cost is detectable when competing against $E. coli$ Ara⁺/Ara⁻ strains encoding $bla_{TEM-1}$.

SME-1 β-lactamase is normally encoded on the chromosome of rare $S. marcescens$ strains (NAAS et al. 1994) and is positively regulated by a LysR family protein, SmeR (NAAS et al. 1995). Cloning the $bla_{SME-1}$ and $bla_{TEM-1}$ genes into constructs with identical promoters, ($P_{trc}$ in pTP123, $P_{lac}$ in pUC18-Kn and $P_{bla}$ in the chromosomal constructs) allows direct comparisons of fitness costs mediated by the gene products but excludes the contribution of transcriptional regulation under native conditions. Although beyond the scope of this work, it would be interesting to investigate the impact of $bla_{SME-1}$ on $S. marcescens$ S6 fitness. The carbapenemase most closely related to SME-1, IMI-2, is to our knowledge the only plasmid-encoded carbapenemase that is inducible by a $cis$-encoded LysR-type regulator (AUBRON et al. 2005). Dissemination of the $bla_{SME-1}$ gene to other bacterial species may be contingent upon gene copy number and the presence of transcriptional regulatory factors.

The widespread dissemination of the TEM-1 β-lactamase among gram negative bacteria exemplifies the robust nature of the $bla_{TEM-1}$ gene and, by extension, has lead to the assumption that β-lactamases do not carry fitness costs in general. However,
evidence for a fitness cost associated with expression of the *Enterobacter cloacae* AmpC gene in *Salmonella enterica* serotype Typhimurium (MOROSINI et al. 2000), the CMY-7 gene in *Salmonella typhimurium* strain LT2 (HOSSAIN et al. 2004) and, here, expression of the *Serratia marcescens* S6 SME-1 gene in *E. coli* suggests that general statements cannot be made concerning compatibility of β-lactamase genes among even closely related bacterial species. A closer examination of the causes for these fitness costs may offer better insight into and perhaps aid in the prevention of β-lactamase dissemination.

The SME-1 enzyme is relatively isolated among class A β-lactamases with respect to its ability to hydrolyze carbapenems. It has been proposed that structural similarities between both substrate (the β-lactam antibiotics vs. D-Ala-D-Ala peptidoglycan side chain) and enzyme (class A and C β-lactamases vs. DD-peptidases) could indicate an *in vivo* interaction of β-lactamases with cell-wall components (BISHOP and WEINER 1992; MOROSINI et al. 2000). Marginal peptidase activity of β-lactamases as well as β-lactamase activity of DD-peptidases has been described *in vitro* (RHAZI et al. 1999). The potential *in vivo* association of SME-1 with cell-wall components provided the impetus for an investigation of the effect of the SME-1 carbapenemase activity upon *E. coli* fitness. As determined by the inability of the catalytically inactivated S70A:E166Q SME-1 mutant to suppress the observed fitness cost, enzymatic activity does not contribute significantly to the fitness cost of SME-1.

The repeated isolation of signal sequence mutations from independent genetic screens suggested a role of the signal sequence in mediating the SME-1 fitness cost. To investigate the role of the SME-1 signal sequence in conferring a fitness cost, hybrid
TEM-SME β-lactamases were constructed. Exchange of SME-1’s native signal sequence for the TEM-1 signal sequence ameliorated the fitness cost of SME-1. Inversely, attaching the SME-1 signal sequence to the mature TEM-1 coding sequence imparted significant toxicity to this normally benign β-lactamase in the form of reduced culture viability, impaired fitness and plasmid loss in *E. coli*. This implicates the SME-1 signal sequence as a source of the SME-1 mediated fitness cost.

Previous studies have described the ability of the signal peptides derived from colicin lysis proteins to reduce growth and viability in *E. coli*, even in the absence of the mature coding region of the lysis protein (KANOH *et al.* 1991; VAN DER WAL *et al.* 1992; VAN DER WAL *et al.* 1994). The enhanced stability associated with the colicin lysis protein’s signal sequences may sequester some component of the protein translocation machinery. Alternatively, the accumulation of these signal sequences may result in pore formation in the cytoplasmic membrane leading to decreased cell viability. Biochemical analysis of the EJh holin, utilized by EJ-1 phage to escape the host cell, revealed that oligomerization of a single transmembrane α-helix is sufficient to generate transmembrane pores (HARO *et al.* 2003). The SME-1 signal sequence may be affecting *E. coli* in a similar manner. Further mutagenic analysis of the SME-1 β-lactamase may offer insight into how the signal sequence influences the deleterious effect of SME-1 in *E. coli*.

The continuing erosion of available antibiotics treatment options has spurred interest in seeking novel antibiotic targets and compounds. Maintenance of existing and future therapies may be facilitated by defining mechanisms of resistance and barriers to horizontal transfer of these resistance elements. The identification of a SME-1 mediated
fitness cost allows the direct application of genetic techniques that have been utilized to understand structural features of β-lactamase function and evolution. The ability to utilize β-lactamase as a model system offers a means to investigate structural determinants of fitness costs and may lead to a broader understanding of how fitness costs affect the horizontal transfer of resistance genes across genera of bacteria.
Acknowledgements: We would like to thank Fahd Majiduddin for creating the pTP123-SME-1 E166Q clone from which the pTP123-SME-1 S70A:E166Q construct was derived. We would also like to thank Ji Yuan and Tulin Ayvaz for technical assistance. The *E. coli* B Ara⁺ and Ara⁻ strains were provided by Richard Lenski. This work was supported by National Institutes of Health grant AI32956 to T. P.


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TABLE 1
Primers used for creating constructs

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<tr>
<th>Primer name</th>
<th>Sequence</th>
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<td>SME-STARTdel</td>
<td>5'-CAATGACAGTTAATAGTAAAGTTACTAGTAACAAAGTAAAAATTAAAAAAAAACGGCTAGC-3'</td>
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<td>SME-Sac</td>
<td>5'-GGGCCTGCTAATCTTACAAACTCAGTC-3'</td>
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<tr>
<td>SME-BamHI</td>
<td>5'-GGGGCCGGATCCCGTCAGGCGCCACAGTC-3'</td>
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<td>SME-C69V-Top</td>
<td>5'-GCCGTTCCCTTTAAGTCTCAAGCTTTTTTTGCGGCTGC-3'</td>
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<td>SME-C238V-Top</td>
<td>5'-GGTGACAAAAACTCGTGGCGCTGGAATACACTGC-3'</td>
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<tr>
<td>SME-E166Q-Top</td>
<td>5'-GGTTAGATCGCTGGGAGCGTGGCGGCTGACACTGC-3'</td>
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<td>SME-1STOP-pyrF384bot</td>
<td>5'-GGCTTCCCTTGCTGCAACTTGCTTAAATCATTGCTGAATT-3'</td>
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*Engineered restriction sites utilized for cloning and/or screening are underlined.*
### Table 2.
Mutant SME-1 genes selected in the presence or absence of ampicillin

<table>
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<tr>
<th>Spontaneous</th>
<th>5'UTR</th>
<th>Signal sequence</th>
<th>Mature sequence</th>
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<td>F19*(^2)</td>
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</tr>
<tr>
<td>Cp.2 (2)(^3)</td>
<td></td>
<td>IS1(^4)</td>
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<tr>
<td>Cp.3</td>
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<td>IS1</td>
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</tr>
<tr>
<td>Cp.4</td>
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<td>IS1</td>
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<td>CpAp.1(^5) (4)</td>
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<td>N20Y</td>
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<tr>
<td>CpAp.2 (2)</td>
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<td>IS5</td>
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<td>CpAp.3</td>
<td></td>
<td>IS10</td>
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<tr>
<td>Cp.5</td>
<td>S2P</td>
<td>K273E</td>
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<td>Cp.6</td>
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<td>L14*</td>
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<td>Δa73</td>
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<td>K4R</td>
<td>F19L</td>
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<td>CpAp.7</td>
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<td>F19L</td>
<td>S62P</td>
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<tr>
<td>CpAp.8 (2)</td>
<td>t121c a124g</td>
<td>F19L</td>
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</tr>
</tbody>
</table>

\(^1\)Cp clones isolated under chloramphenicol selection.
\(^2\)STOP codon represented by *.
\(^3\)Clones isolated multiple times indicated by number in parenthesis.
\(^4\)Occurance of insertion sequence as described in text.
\(^5\)CpAp clones isolated under chloramphenicol + 100 µg/ml ampicillin selection.
**TABLE 3**

Effect of the SME-1 gene and its derivatives on *E. coli* B fitness

<table>
<thead>
<tr>
<th>Competition experiment</th>
<th>Selection rate constant (day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTP123-SME-1 vs pTP123</td>
<td>-10.7 ± 0.7***</td>
</tr>
<tr>
<td>pTP123-SME-1 vs pTP123-TEM-1</td>
<td>-7.0 ± 2.4***</td>
</tr>
<tr>
<td>chSME-1 Ara(^+) vs chTEM-1 Ara(^+)</td>
<td>-1.3 ± 0.5***</td>
</tr>
<tr>
<td>chSME-1 Ara(^-) vs chTEM-1 Ara(^-)</td>
<td>-0.2 ± 0.1**</td>
</tr>
<tr>
<td>pTP123-SME-1 vs Trunc. SME-1</td>
<td>-2.2 ± 1.3***</td>
</tr>
<tr>
<td>pTP123-SME-1 vs SME(_{\Delta})START</td>
<td>-3.8 ± 2.8***</td>
</tr>
<tr>
<td>SME(_{\Delta})START vs pTP123-TEM-1</td>
<td>-0.3 ± 0.7</td>
</tr>
<tr>
<td>pTP123-SME-1 vs SME S70A:E166Q</td>
<td>-0.9 ± 1.4</td>
</tr>
<tr>
<td>pTP123-SME-1 vs SME C69V:C238V</td>
<td>1.4 ± 1.0*</td>
</tr>
<tr>
<td>pTP123-SME-1 vs TEM(SS)SME</td>
<td>-3.9 ± 1.3***</td>
</tr>
<tr>
<td>TEM(SS)SME vs pTP123-TEM-1</td>
<td>-0.9 ± 1.2</td>
</tr>
<tr>
<td>SME(SS)TEM vs pTP123-TEM-1</td>
<td>-1.4 ± 1.1**</td>
</tr>
<tr>
<td>pTP123-SME-1 vs SME(SS)TEM</td>
<td>-4.4 ± 1.2***</td>
</tr>
</tbody>
</table>

\(^1\) A negative selection rate constant is indicative of a fitness cost associated with the initial construct (e.g. SME-1) relative to the construct it is competing against (e.g. TEM-1). Significance based on paired *t* test: *, 0.05<P<0.01; **, 0.01<P<0.001; ***, P<0.001.
**FIGURE LEGENDS**

**Figure 1:** Effect of β-lactamase genes on *E. coli* growth rates. A) Growth curves in LB broth of *E. coli* K12 XL1-Blue containing either pTP123, pTP123-TEM-1 or pTP123-SME-1 were obtained by monitoring OD$_{600}$ over time. B) Growth curves of a frame-shifted SME-1 clone and point mutants that eliminate enzymatic activity (S70A:E166Q) or disulfide bond formation (C69V:C238V). C) Growth curves of chimeric constructs in which signal sequences of TEM-1 and SME-1 have been exchanged. Error bars represent standard deviation of at least three independent experiments.

**Figure 2:** Effect of β-lactamase genes on culture viability. The number of colony forming units per ml is indicative of culture viability. Data for *E. coli* K12 XL1-Blue containing the indicated plasmids was normalized to *E. coli* K12 XL1-Blue harboring isogenic plasmid without a β-lactamase gene. Two genetic contexts (pTP123 parent and pUC18-Kn parent plasmids) were examined. Error bars represent the standard deviation of at least six independent experiments. Significance based on *t*-test against empty vector: ***, P<0.001.

**Figure 3:** Plasmid loss in the absence of antibiotic selection. Overnight cultures were spread onto LB agar plates and LB agar plates containing chloramphenicol to determine the percentage of cells harboring plasmid. Errors bars represent the standard deviation for at least two independent experiments.
Figure 1:

A

B

C
Figure 2:
Figure 3: