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Selection of hyperproduction of AmpC and SME-1 in a carbapenem-resistant *Serratia marcescens* isolate during antibiotic therapy

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Objectives: Antibiotic selective pressure may result in changes to antimicrobial susceptibility throughout the course of infection, especially for organisms that harbour chromosomally encoded AmpC β-lactamases, notably *Enterobacter* spp., in which hyperexpression of *ampC* may be induced following treatment with cephalosporins. In this study, we document a case of bacteraemia caused by a bla_{SME-1}-harbouring *Serratia marcescens* that subsequently developed resistance to expanded-spectrum cephalosporins, piperacillin/tazobactam and fluoroquinolones, over the course of several months of treatment with piperacillin/tazobactam and ciprofloxacin.

Methods: Susceptibility testing and WGS were performed on three *S. marcescens* isolates from the patient. β-Lactamase activity in the presence or absence of induction by imipenem was measured by nitrocefin hydrolysis assays. Expression of *ampC* and bla_{SME-1} under the same conditions was determined by real-time PCR.

Results: WGS demonstrated accumulation of missense and nonsense mutations in *ampD* associated with stable derepression of AmpC. Gene expression and β-lactamase activity of both AmpC and SME-1 were inducible in the initial susceptible isolate, but were constitutively high in the resistant isolate, in which total β-lactamase activity was increased by 128-fold.

Conclusions: Although development of such *in vitro* resistance due to selective pressure imposed by antibiotics is reportedly low in *S. marcescens*, our findings highlight the need to evaluate isolates on a regular basis during long-term antibiotic therapy.

Introduction

De novo selection of antibiotic resistance through the course of antibiotic therapy is a well-documented event for Gram-negative bacteria that possess chromosomally encoded and inducible AmpC β-lactamases.[1] Notably, bacteraemia caused by *Enterobacter* spp. treated with an expanded-spectrum cephalosporin can lead to selection of mutants with derepressed expression of the *ampC* gene and clinical failure.[2,3] Therefore, many recommend cefepime or a carbapenem for the treatment of AmpC-producing bacteria, as these β-lactams are poorly hydrolysed by AmpC.[4] However, carbapenemase-producing Enterobacteriaceae resistant to both cefepime and carbapenems are widespread throughout the world, resulting in few or no treatment options for these organisms.[5] In the USA, KPC is the most prevalent carbapenemase, although others, such as SME, are sporadically reported.[6]

The SME carbapenemases are class A serine β-lactamases, identified, to date, only in *Serratia marcescens*. In contrast to other serine carbapenemases, SME enzymes are phenotypically unique, exhibiting poor hydrolytic activity against expanded-spectrum cephalosporins.[7–9] As such, isolates that harbour SME often test susceptible to ceftazidime or ceftriaxone, but resistant to ertapenem, imipenem, meropenem and aztreonam.[8–10] In addition, bla_{SME} is chromosomal; isolates producing the enzyme do not typically express the same MDR profile seen in isolates with plasmid-mediated carbapenemases such as bla_{KPC}, which usually harbour genes conferring resistance to other antimicrobial classes on the same plasmid. To date, five SME variants have been described, SME-1 to SME-5 (NCBI, ftp://ftp.ncbi.nlm.nih.gov/pathogen/betalactamases/Allele.tab).

Herein, we document a case of SME-producing *S. marcescens* bacteraemia. The isolate was initially susceptible to ciprofloxacin,

expanded-spectrum cephalosporins and piperacillin/tazobactam, and the patient was treated with piperacillin/tazobactam and ciprofloxacin. Over the course of several months, the patient's isolates became resistant to these two agents *in vivo*. WGS elucidated the origins of this resistance and phenotypic, biochemical and gene expression studies demonstrated enhanced activity of both AmpC and SME enzymes in the resistant isolates.

Patients and methods

Case summary

A patient in their early forties with a history of alcoholic cirrhosis, oesophageal varices and hepatic encephalopathy presented to an outside hospital with nausea and haematemesis. The patient was haemodynamically unstable with an international normalized ratio of 10 and transferred to our institution for liver transplantation evaluation. Oesophagogastroduodenoscopy upon admission demonstrated gastric stricture. The patient remained haemodynamically stable until hospital day (HD) 7, when recurrent haematemesis and hypotension prompted banding of oesophageal varices. Ceftriaxone was started after the procedure and blood culture on HD 7 grew *S. marcescens* (isolate D-1) resistant to ertapenem, imipenem and meropenem, and susceptible to ceftriaxone, cefepime and piperacillin/tazobactam (Table 1). Therapy was switched to piperacillin/tazobactam on HD 8 and was continued until liver transplantation on HD 26. Fever in association with T-tube dislodging prompted repeat blood cultures on HD 39 and a biliary repair procedure on HD 41. Blood cultures from HD 39 again grew *S. marcescens* (isolate D-2; Table 1) and the patient was treated with 15 days of piperacillin/tazobactam, adjusted for renal dysfunction (2.25 g intravenously q6h) and oral ciprofloxacin for 7 days. The patient developed delirium and repeated emesis on HD 90, which prompted a culture of the patient's Jackson–Pratt drain fluid, which yielded *S. marcescens* isolate D-3 (Table 1), resistant to trimethoprim/sulfamethoxazole, ciprofloxacin and piperacillin/tazobactam. This isolate was deemed a colonizer and the patient was not treated. The patient was discharged to a skilled nursing facility on HD 125 and readmitted 13 days later for fever, hypotension and shortness of breath. The patient was intubated, started on two vasopressors, continuous renal replacement therapy and vancomycin and piperacillin/tazobactam. Blood cultures grew *Escherichia coli* and amikacin was added. Admission respiratory cultures grew *S. marcescens* and repeat blood cultures on HD 2 and HD 4 also grew *S. marcescens*. All *S. marcescens* isolated during this second hospitalization had identical susceptibility profiles to D-3 (not shown). Despite continued therapy with amikacin, ciprofloxacin and piperacillin/tazobactam, the patient's clinical condition deteriorated and the patient died on the sixth day of their second admission. The timeline of the patient's hospital course and isolation of each *S. marcescens* isolate is summarized in Figure 1.

Methods

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by broth microdilution in CAMHB, according to CLSI standards,[11] using panels prepared in-house. Antimicrobial susceptibility results were interpreted according to CLSI M100S 2017 breakpoints.[11]

WGS

Isolates D-1, D-2 and D-3 were screened for the presence of bla_{SME} by PCR amplification with published primer sets[6,8] (Table S1, available as Supplementary data at *JAC* Online) then subjected to WGS, as described elsewhere.[12] Briefly, genomic DNA was prepared using EZ1 Biorobot with DNA Tissue Kit (QIAGEN, Valencia, CA, USA). DNA sequencing was performed on an Illumina MiSeq platform (2×250 bp reads). Sequencing coverage among all isolates was between 140× and 200×. Illumina reads were assembled *de novo* using the A5 Pipeline[13] into larger contigs, which were annotated using RAST[14] and PATRIC.[15,16] Raw reads were processed on a Galaxy server[17] using FASTQ Groomer,[18] followed by FASTQ Joiner[18] to match and merge paired reads. The CSI Phylogeny tool[19] was used to identify high-quality SNPs between isolates. Variant call format (VCF) files from CSI Phylogeny output were combined using the VCFcombine tool[20] on the Galaxy server. The combined VCF file was analysed and visualized using VariationBrowser software.[21] Whole genome alignment was performed on annotated merged contigs using progressive Mauve[22] and genomic differences and rearrangements were visualized using Geneious version 10.[23] WGS data were submitted to GenBank under BioProject accession number PRJNA381643 (BioSample accession numbers SAMN06685768, SAMN06685769 and SAMN06685770 for isolates D-1, D-2 and D-3, respectively).

Table 1. Antimicrobial susceptibility of isolates in this study as determined by the reference broth microdilution method

	Isolate(s)	
	D-1 and D-2	D-3
Specimen source (HD isolated)	blood (7 and 39)	bile drainage (90)
MIC (mg/L) (interpretation)		
aminoglycosides		
AMK	2 (S)	1 (S)
GEN	≤0.5 (S)	≤0.5 (S)
TOB	1 (S)	≤0.5 (S)
cephalosporins		
FEP	≤0.5 (S)	1 (S)
CAZ	≤0.5 (S)	2 (S)
CRO	≤0.5 (S)	16 (R)
fluoroquinolones		
CIP	≤0.5 (S)	>2 (R)
LVX	≤2 (S)	>8 (R)
β-lactam/β-lactamase inhibitors		
TZP	16/4 (S)	128/4 (R)
carbapenems		
ETP	>4 (R) (endpoint 8)	>4 (R) (endpoint 16)
IPM	>8 (R) (endpoint 128)	>8 (R) (endpoint 64)
MEM	>16 (R) (endpoint 16)	16 (R) (endpoint 16)
DOR	8 (R)	8 (R)
folate pathway inhibitors		
SXT	≤1/20 (S)	2/38 (S)

AMK, amikacin; GEN, gentamicin; TOB, tobramycin; FEP, cefepime; CAZ, ceftazidime; CRO, ceftriaxone; CIP, ciprofloxacin; LVX, levofloxacin; TZP, piperacillin/tazobactam; ETP, ertapenem; IPM, imipenem; MEM, meropenem; DOR, doripenem; SXT, trimethoprim/sulfamethoxazole; R, resistant; S, susceptible.

SME and AmpC induction and measurement of β-lactamase activity

S. marcescens D-1 and D-3 were grown overnight, then diluted 1:20 in LB broth and grown for 2 h to reach log phase. Aliquots of D-1 and D-3 were treated for 2 h with either imipenem as an inducer at a final concentration of 10 mg/L[24] or with 0.02 M phosphate buffer, pH 7.0 (PB) as control. This inducing imipenem concentration was based on a previous study of the role of AmpD on regulation of expression of β-lactamases NmcA and AmpC

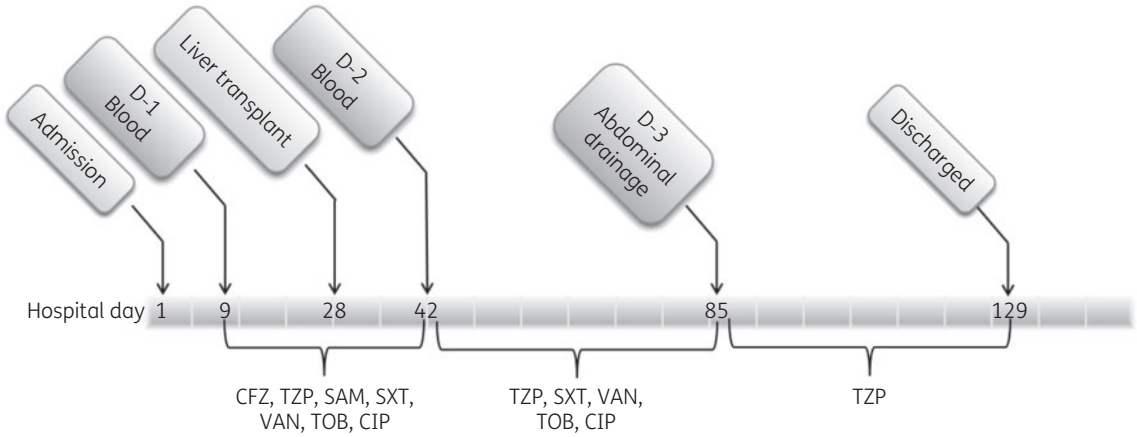

Figure 1. Timeline of admission, with collection date for studied isolates and antibiotics used in the interval between isolations of *S. marcescens*. CFZ, cefazolin; TZP, piperacillin/tazobactam; SAM, ampicillin/sulbactam; SXT, trimethoprim/sulfamethoxazole; VAN, vancomycin; TOB, tobramycin; CIP, ciprofloxacin.

in *Enterobacter cloacae*[24] and the plasma concentration of imipenem at 2 h post-infusion in healthy volunteers.[25] As a positive control, the AmpC-producing *S. marcescens* isolate SC 9782 in an SME-negative background[26] was induced with cefoxitin (10 mg/L in PB) or treated with PB as a reference. After induction, centrifuged cell pellets were washed with PB; pelleted cells were suspended in 0.2 M acetate buffer, pH 5.5.

β-Lactamases were extracted from the cells following five freeze/thaw cycles (dry ice–ethanol bath/37°C water bath). The clarified supernatant containing periplasmic contents, including β-lactamases, was tested for β-lactamase activity. Protein concentrations were measured using the bicinchoninic acid (BCA) assay (Pierce BCA Protein Assay Kit, Thermo Fisher, Waltham, MA, USA). β-Lactamase activity in each extract was measured using a nitrocefin hydrolysis assay. To differentiate SME and AmpC activities, extracts were incubated for 2 min with either PB, to determine total β-lactamase activity (the sum of SME and AmpC activities), or 15 mg/L aztreonam, a concentration that inhibited AmpC activity by at least 95% under the conditions tested. Aztreonam is a potent AmpC inhibitor[27] with poor affinity for SME.[9] Prewarmed nitrocefin (500 mg/L in PB) was added to each extract after the incubation. Hydrolysis rates were measured at 490 nm with a BioTek5 plate reader (BioTek, Winooski, VT, USA) for 1–2 min using only the initial linear rate of reaction to determine nitrocefin hydrolysis/min/ng of protein in triplicate assays. Hydrolysis rates corresponding to AmpC and SME activities were calculated from the nitrocefin hydrolysis rates of cell extracts in the presence and absence of aztreonam.

AmpC and SME transcription analysis

Colonies from overnight cultures of isolates D-1 and D3 were resuspended in 4 mL of LB broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) to a turbidity equivalent to that of a 1.0 McFarland standard and incubated at 35°C on a tube rocker for 2 h. Expression of AmpC-type β-lactamase genes bla_{SRT-2} and bla_{SME-1} was induced using imipenem for 2 h, a timepoint at which the *ampC* mRNA level in *E. cloacae* reaches its peak after induction with cefoxitin,[28] as described above. After induction, 200 μL of RNAProtect Bacteria Reagent (QIAGEN) was added to 100 μL of broth culture, followed by centrifugation at 5000 **g** for 10 min. RNA was extracted on the QIAcube platform (QIAGEN) using the RNeasy Mini Kit (QIAGEN). DNase treatment was performed using the TURBO DNA-free Kit (Life Technologies, Grand Island, NY, USA). RNA integrity and quantity were evaluated using the Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA). cDNA synthesis with SuperScript IV VILO Master Mix (Life Technologies) was performed on the GeneAmp PCR System 9700 (Life Technologies) with 1 μg of total RNA. Incubation time at 50°C was 20 min to maximize cDNA yields according to the manufacturer's instructions. Relative expression of *ampC*, bla_{SME} and *rpoB* was quantified using real-time PCR. Each 20 μL PCR included 10 μL of TaqMan Fast Advanced Master Mix (Life Technologies), *S. marcescens* bla_{SRT-2}-, bla_{SME-1}- and *rpoB*-specific primers and probes (Table S1) at final concentrations of 500 and 250 nM, respectively, and 5 μL of diluted cDNA or a no-reverse transcriptase control to detect genomic DNA contamination. PCR was performed on a LightCycler® 480 (Roche Diagnostics, Indianapolis, IN, USA) with the following settings: cDNA synthesis at 50°C for 2 min and initial denaturation at 95°C for 20 s, followed by 40 two-step cycles of 95°C for 3 s and 60°C for 30 s. Fluorescence was detected after the second step of each cycle. PCR efficiency for each target gene was determined using six 10-fold dilutions of cDNA and was used to calculate efficiency-adjusted ratios between bla_{SRT-2} and bla_{SME-1} to *rpoB* using the Advance Relative Quantification Module of the LightCycler® 480 software (version 1.5.0 SP3; Roche Diagnostics).[29] Fold changes in gene expression between imipenem-induced and non-induced samples were calculated from these normalized ratios. Statistical analysis was performed in BioStat version 6 software[30] using two-tailed one-sample Student's *t*-test with a hypothetical mean of 1. The basal expression of both bla_{SRT-2} and bla_{SME-1} in D-3 compared with D-1 was calculated from efficiency-adjusted Ct values using the ΔΔCt method, followed by statistical analysis using unpaired Student's *t*-test.

Results

Susceptibility profiles of the isolates investigated are shown in Table 1. WGS performed on the isolates revealed bla_{SME-1} was located on a 28.4 kb genomic island called SmarGI1-1[31] (Figure 2), the only acquired antimicrobial resistance gene identified. In addition, all isolates harboured a bla_{SRT-2} AmpC-type β-lactamase gene. Comparative genomics of the isolates revealed the chromosomes of D-1 and D-2 were identical, with five non-conservative SNPs between these two isolates and isolate D-3 (Table 2). Notably, a predicted mutation H61P was found in *ampD* and a mutation predicted to encode an early stop codon at position 226 was identified in another protein with the same putative function as *ampD* (Table 2). Mutations predicted to encode S83R in the DNA gyrase subunit A gene *gyrA* and G60A in the transcriptional repressor *sdeS* were identified. The S83R *gyrA* mutation has been associated with fluoroquinolone resistance in *S. marcescens*,[32,33]

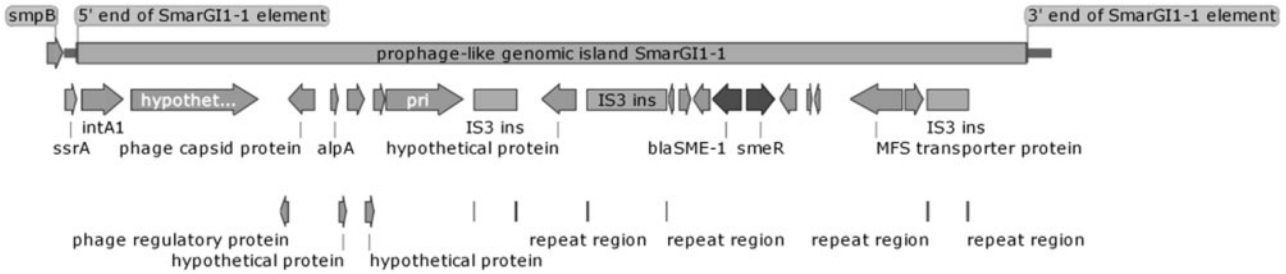

Figure 2. Chromosomal genomic island containing bla_{SME-1} identified by WGS in all three isolates.

Table 2. Mutations identified between the genomes of isolates D-1/D-2 and D-3

Gene name	NCBI locus tag in isolate D-1	Putative function	Single nucleotide change	Gene position	Predicted amino acid	
					isolates D-1 and D-2	isolate D-3
betB	B7L73_09145	betaine aldehyde dehydrogenase	T>G	128	I	S
sdeS	B7L73_11155	transcriptional repressor of RND-family SdeAB efflux pump	G>C	60	G	A
gyrA	B7L73_12815	DNA gyrase subunit A	C>A	83	S	R
ampD (putative)	B7L73_13205	*N*-acetylmuramoyl-L-alanine amidase	C>T	226	Q	STOP
ampD	B7L73_13755	*N*-acetylmuramoyl-L-alanine amidase	T>G	61	H	P

while mutations in *sdeS* are associated with overexpression of the SdeAB multidrug efflux pump,[34] whose main substrates include ciprofloxacin and chloramphenicol, but not β-lactams.[35]

β-Lactamase activity in D-1 and D-3 was evaluated in the presence and absence of imipenem, which induces AmpC activity, and aztreonam, which inhibits AmpC activity[27] (Figure 3). In extracts from the SME-negative *S. marcescens* strain SC 9782, aztreonam inhibited AmpC activity by 96%. Total basal (non-induced) β-lactamase activity of D-3 was 128-fold higher than for D-1, with 0.85 (SD ±0.18) μmoles of nitrocefin hydrolysed/min/ng of protein by isolate D-1 compared with 109 (SD ±21.6) μmoles/min/ng by isolate D-3 (Figure 3). The basal activity of SME-1 in D-3 was 75-fold that of D-1, with 0.15 (SD ± 0.0072) μmoles/min/mg by D-1 compared with 11.3 (SD ± 6.7) μmoles/min/mg by D-3 (Figure 3). Basal AmpC activity of D-3 was also elevated, at 140 times that of D-1, consistent with derepression of AmpC regulation (Figure 3). Addition of imipenem as an inducer resulted in a 21.4-fold increase in total β-lactamase activity in D-1 (from basal activity of 0.85 to 18.2 μmoles/min/ng post-induction), but only a 1.29-fold increase in total β-lactamase activity in D-3 post-induction, again consistent with constitutive expression of β-lactamase in D-3 (Figure 3). In D-1, imipenem increased AmpC activity by 25.0-fold (from basal activity of 0.70 to 17.5 μmoles/min/ng post-induction) and SME-1 activity by 4.67-fold (from basal activity of 0.15 to 0.70 μmoles/min/ng post-induction) (Figure 3). In D-3, addition of imipenem resulted in a 1.30-fold increase in AmpC activity (from basal activity of 97.7 to 127 μmoles/min/ng post-induction) and a 1.28-fold increase in SME-1 activity (from basal activity of 11.3 to 14.4 μmoles/min/ng post-induction).

mRNA expression was evaluated for bla_{SRT-2} and bla_{SME-1}, normalized to that of the housekeeping *rpoB* gene. The basal expression of both β-lactamases in D-3 was significantly higher compared with that of D-1, with $\Delta\Delta Ct = -4.72 \pm 0.542$ ($P = 0.001$) for bla_{SRT-2} and -3.53 ± 0.216 ($P < 0.0001$) for bla_{SME-1}. The increased expression of these genes in D-3 compared with D-1 was also observed after imipenem induction, with $\Delta\Delta Ct = -4.36 \pm 0.613$ ($P = 0.0021$) for bla_{SRT-2} and -3.06 ± 0.254 ($P = 0.0003$) for bla_{SME-1}. When compared for imipenem-non-induced and -induced samples (Figure 4), addition of imipenem to D-1 resulted in significant upregulation of bla_{SRT-2} and bla_{SME-1} mRNA by 3.29 ± 0.62-fold ($P = 0.0233$) and 2.72 ± 0.38-fold ($P = 0.0155$) that of non-induced samples. In contrast, imipenem induction did not result in a significant increase in bla_{SRT-2} or bla_{SME-1} ($P = 0.45$) expression in D-3, when evaluated by one-sample two-tail *t*-test ($P = 0.44$ and $P = 0.45$, respectively).

Discussion

Antimicrobial resistance is an evolutionary process that involves adaptation to the changing environment.[36] Selective pressure imposed by antibiotic therapy can lead to intra-host adaptation, including accumulation of *de novo* mutations or acquisition of genetic elements that confer resistance to antibiotics, providing an evolutionary advantage to the resistant population.[37,38] There have been a limited number of longitudinal studies that utilized both phenotypic testing and WGS to examine temporal acquisition of antimicrobial resistance among Enterobacteriaceae isolated from patients undergoing antibiotic treatment.[39,40] Herein, we demonstrated emergence of ceftriaxone and piperacillin/tazobactam resistance, as a result of AmpC and SME-1 derepression in a case of carbapenem-resistant *S. marcescens* bacteraemia treated with piperacillin/tazobactam. Derepression of AmpC in *S. marcescens*

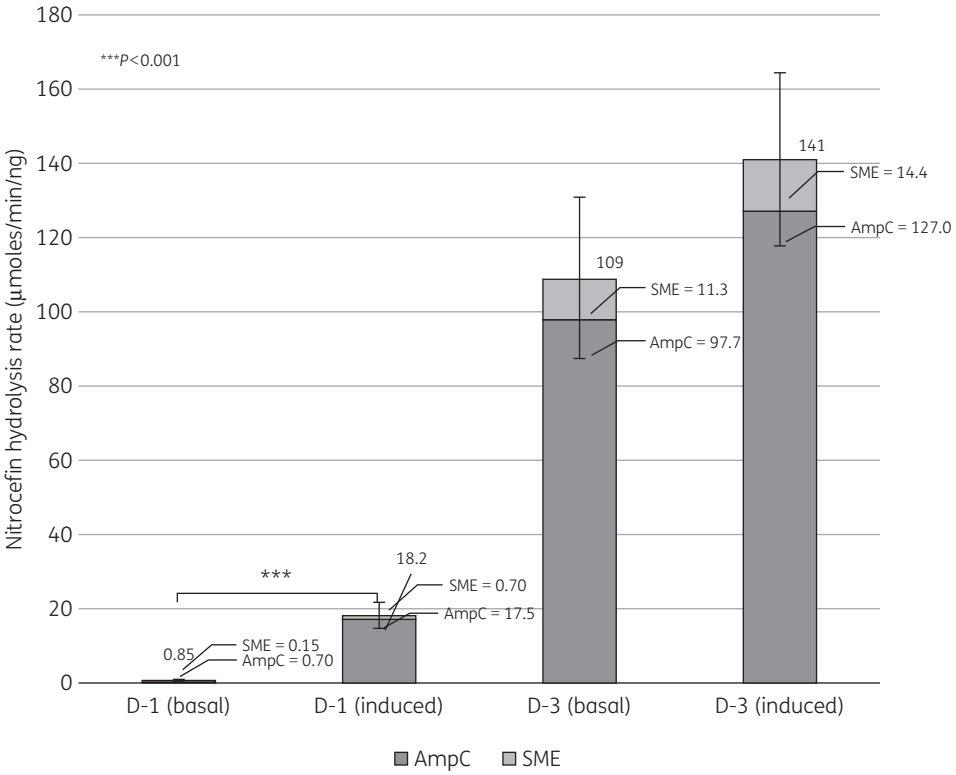

Figure 3. Total β-lactamase activity before and after induction with 10 mg/L IPM in *S. marcescens* D-1 and D-3. Rates of nitrocefin hydrolysis were measured in μmoles of nitrocefin hydrolysed/min/ng of protein in cell extracts. Results represent the mean of total β-lactamase activity (±SD) ($n = 3$). The mean values for AmpC and SME activity contributing to total β-lactamase activity (based on 96% inhibition of AmpC activity by ATM) are also represented in the stacked bars. IMP, imipenem; ATM, aztreonam.

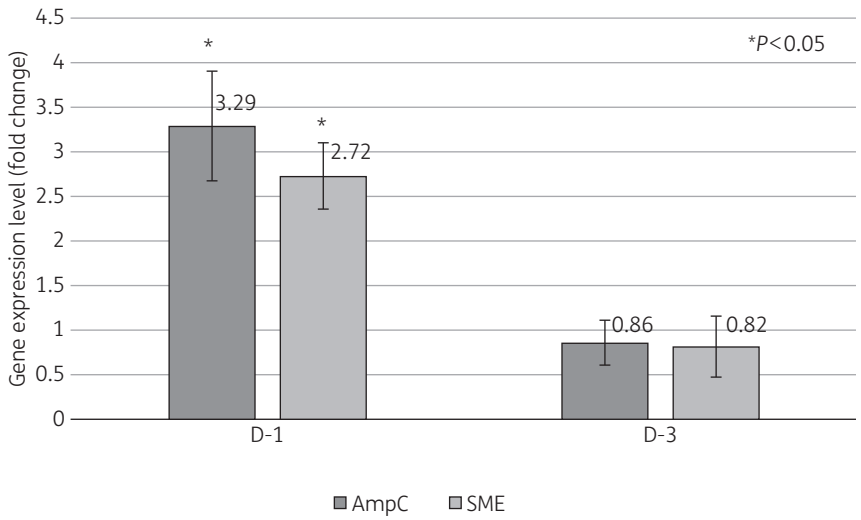

Figure 4. Upregulation of *ampC* and bla_{SME-1} gene expression in isolates D-1 and D-3 after induction by IPM. Results represent the mean fold change (±SD) for each gene ($n = 3$) compared with a non-induced sample. *P* values are in comparison with the theoretical mean of 1.0. IMP, imipenem.

during treatment is reportedly low (0%–7%),[3,24,41] in particular when compared with *Enterobacter* spp., in which emergence of resistance is more common (8.3%–19%).[3,42–44] piperacillin/tazobactam has infrequently been associated with selection of resistance due to derepression of chromosomal AmpC in *S. marcescens*,[3,41] but is the most likely cause for selection of stably derepressed mutants herein. Although piperacillin and ceftriaxone are poor substrates for the AmpC and SME-1 enzymes, they will be affected by derepression

of the two enzymes.[45] Thus, this case demonstrates a clinical challenge, as the isolate was resistant to the carbapenems as a result of its SME-1 enzyme, leaving few treatment options.

Unsurprisingly, gene expression and β-lactamase activity studies demonstrated a constitutive, high-level expression of *ampC* (bla_{SRT-2}) in isolate D-3, the isolate in which we identified *ampD* mutations; in addition, we observed a similar phenotype for bla_{SME-1}, which may also be associated with an *ampD* mutation. The *ampD* gene encodes a cytoplasmic amidase that hydrolyses muropeptides, which accumulate after exposure to β-lactams and bind the regulatory protein AmpR, resulting in increased expression of *ampC*.[46] Conversely, *ampD* expression decreases the intracellular concentration of muropeptide, leading to downregulation of *ampC* expression.[47,48] Mutations in the *ampD* gene that affect its amidase function are the most common cause of constitutive hyperproduction of AmpC, due to accumulation of muropeptides in the cytoplasm.[1] Regulation of SME gene expression is less well understood. A LysR-family transcriptional regulator SmeR, located directly upstream to the bla_{SME-3} gene, has been shown to be a weak inducer of bla_{SME-3} in the presence of imipenem.[49] However, the entire genomic island carrying bla_{SME-1} and *smeR* were identical for all isolates in this study, suggesting that the constitutively high levels of bla_{SME-1} expression and activity in D-3 were due to elements outside this genomic island. The SmeR amino acid sequence shares 66% sequence identity to that of NmcR, also a LysR-family transcriptional regulator first identified in *E. cloacae*.[24,49] NmcR regulates the expression of *nmcA*, a gene that encodes a serine carbapenemase found in *E. cloacae*. Interestingly, mutations in *ampD* were also associated with derepression of NmcA.[24] Considering the homology between *smeR* and *nmcR*, it is possible that the *ampD* mutations in D-3 resulted in derepression of SME-1. However, this hypothesis requires further confirmation.

In conclusion, our study described an unusual temporal acquisition of resistance to expanded-spectrum cephalosporins in *S. marcescens* isolates from a single patient during the course of antimicrobial treatment. Although emergence of *in vivo* resistance to expanded-spectrum cephalosporins during treatment of *S. marcescens* infection has been reportedly low, clinical microbiology laboratories and physicians should be aware of this possibility and perform susceptibility testing on sequential *S. marcescens* isolates to detect resistance that may have developed over the course of antimicrobial treatment.[11]

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Transparency declarations

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Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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