J Antimicrob Chemother 2011; **66**: 2033–2037
doi:10.1093/jac/dkr276 Advance Access publication 30 June 2011

Journal of Antimicrobial Chemotherapy

Inactivation of *mrcA* gene derepresses the basal-level expression of L1 and L2 β-lactamases in *Stenotrophomonas maltophilia*

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Received 26 December 2010; returned 20 May 2011; revised 30 May 2011; accepted 8 June 2011

Objectives: To characterize the relationship between inactivation of the *mrcA* gene and β-lactamase expression and β-lactams resistance in *Stenotrophomonas maltophilia* KJ and to investigate the involvement of *ampR*, *ampN-ampG*, *ampD$_I$* and *creBC* in this.

Methods: The mrcA deletion mutant KJΔmrcA was constructed to investigate the role of this putative penicillin-binding protein 1a (PBP1a) in β-lactamase expression and β-lactam resistance. The *ΔampR*, *ΔampNG*, *ΔampDI* and *ΔcreBC* alleles were introduced into KJΔmrcA, and KJΔDIΔBC and KJΔDIΔmrcAΔBC were also constructed for comparison. All the mutants and their corresponding parent strains were assayed for β-lactamase activities and MICs of β-lactams.

Results: Inactivation of *mrcA* caused basal L1/L2 β-lactamase production to increase by ~100-fold, but made little difference to cefuroxime-induced β-lactamase activity and the MICs of β-lactams. The Δ*mrcA*-derived basal β-lactamase hyperproduction was *ampR* and *ampN-ampG* dependent. Simultaneous inactivation of *ampD$_I$* and *mrcA* did not augment β-lactamase production over and above that seen in an *ampD$_I$* mutant alone. Furthermore, we could find no evidence for a role of the *creBC* two-component regulatory system in β-lactamase hyperproduction in a Δ*ampD$_I$* or Δ*mrcA* background.

Conclusions: Inactivation of *mrcA*, predicted to encode PBP1a, causes basal L1/L2 β-lactamase hyperproduction in *S. maltophilia*.

Keywords: *S. maltophilia*, penicillin-binding proteins, PBPs

Introduction

Peptidoglycan determines bacterial cell shape and protects the bacterium from osmotic lysis. Penicillin-binding proteins (PBPs) are a set of membrane-bound enzymes participating in the final stages of peptidoglycan biosynthesis. Each bacterium has a unique set of PBPs. There are 13 known PBPs in *Escherichia coli*, being PBPs 1a, 1b, 1c, 2, 3, 4, 4b, 5, 6 and 7, DacD, as well as the non-membrane-associated AmpC, and AmpH.[1]

In some members of the family Enterobacteriaceae, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*, the production of chromosomal β-lactamase is induced during β-lactam challenge, and this is the major mechanism of β-lactam resistance. The *ampR-ampC* module of *P. aeruginosa* is a well-known induction model typified by that characterized in *Citrobacter freundii*, where AmpC β-lactamase expression is linked to β-lactam-induced changes in cytoplasmic peptidoglycan recycling product concentrations by a mechanism that requires the proteins AmpG and NagZ to import and process the appropriate activatory ligand and the transcriptional regulator AmpR, to which the activatory ligand binds. The AmpD protein is also involved because it degrades the AmpR activatory ligand, repressing AmpC production during normal growth. Loss of AmpD, therefore, 'derepresses' AmpC production, leading to high-level β-lactam resistance.[2] Recently Moya *et al.*[3] described how inactivation of PBP4, encoded by *dacB*, confers *ampC* overexpression and β-lactam resistance in *P. aeruginosa*, with resistance being partly due to activation of the CreBC(BlrAB) two-component regulatory system and partly due to AmpC hyperproduction.

S. maltophilia is inherently resistant to β-lactams via the inducible production of two chromosomally encoded β-lactamases, L1 and L2. The regulation of the L1 and L2 induction in *S. maltophilia* is also AmpR dependent, and appears to be closely coupled with peptidoglycan recycling, as is the AmpC induction in *P. aeruginosa*, except for a few differences: (i) the permease system in *S. maltophilia* involves

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Table 1. Bacterial strains, plasmids and primers used in this study

Strain, plasmid or primer	Genotype or properties	Reference
S. maltophilia		
KJ	wild-type, a clinical isolate from Taiwan	9
KJΔmrcA	*S. maltophilia* KJ *mrcA* deletion mutant; Δ*mrcA*	this study
KJΔL1	*S. maltophilia* KJ *L1* mutant; *L1::xylE*	9
KJΔL1ΔmrcA	*S. maltophilia* KJ *L1* and *mrcA* double mutant; *L1::xylE*, Δ*mrcA*	this study
KJΔL2	*S. maltophilia* KJ *L2* mutant; *L2::xylE*	9
KJΔL2ΔmrcA	*S. maltophilia* KJ *L2* and *mrcA* double mutant; *L2::xylE*, Δ*mrcA*	this study
KJΔNG	*S. maltophilia* KJ *ampN* and *ampG* deletion mutant; Δ*ampN*, Δ*ampG*	this study
KJΔNGΔmrcA	*S. maltophilia* KJ *ampN*, *ampG* and *mrcA* deletion mutant; Δ*ampN*, Δ*ampG*, Δ*mrcA*	this study
KJΔDI	*S. maltophilia* KJ $ampD_I$ deletion mutant; $\Delta ampD_I$	5
KJΔDIΔmrcA	*S. maltophilia* KJ $ampD_I$ and *mrcA* deletion mutant; $\Delta ampD_I$, Δ*mrcA*	this study
KJΔR	*S. maltophilia* KJ *ampR* deletion mutant; Δ*ampR*	this study
KJΔRΔmrcA	*S. maltophilia* KJ *ampR* and *mrcA* deletion mutant; Δ*ampR*, Δ*mrcA*	this study
KJΔDIΔBC	*S. maltophilia* KJ $ampD_I$ and *creBC* deletion mutant; $\Delta ampD_I$, Δ*creBC*	this study
KJΔmrcAΔBC	*S. maltophilia* KJ *mcrA* and *creBC* deletion mutant; Δ*mrcA*, Δ*creBC*	this study
KJΔDIΔmrcAΔBC	*S. maltophilia* KJ *ampDI*, *mcrA* and *creBC* deletion mutant; $\Delta ampD_I$, Δ*mrcA*, Δ*creBC*	this study
E. coli		
DH5α	F- ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_k^- m_k^+) *phoA supE44*λ^- *thi-1 gyrA96 relA1*	Invitrogen
S17-1	*recA pro thi hsdR* with integrated RP4-2-tc::Mu-kan::Tn*7*; Tra^+ Tr^r Sm^r	4
Plasmids		
pEX18Tc	*sacB oriT*, Tc^r	4
pΔmrcA	pEX18Tc with a 1377 bp DNA fragment containing the partial *mrcA* gene with an internal 207 bp deletion; Tc^r	this study
pΔR	pEX18Tc with a 1628 bp DNA fragment containing the complete *ampR* gene with an internal 468 bp deletion; Tc^r	this study
pΔNG	pEX18Tc with a 1998 bp DNA fragment containing the 474 bp upstream of *ampN*, 378 bp 5′ terminal of *ampN*, and 1146 bp 3′ terminal of *ampG*; Tc^r	this study
pΔDI	pEX18Tc with a 1918 bp DNA fragment containing the intact $ampD_I$ gene with an internal 103 bp deletion; Tc^r	5
pΔBC	pEX18Tc with a 1050 bp DNA fragment containing the partial 5′-terminus of *creB* gene and partial 3′-terminus of *creC* gene; Tc^r	this study
Primers		
MrcA-F	5′-GATGCAGCCCCTGACTGTTCC C-3′	this study
MrcA-R	5′-GAAGTTGAAGCCGTCCACCAC C-3′	this study
CreB5-F	5′-AGCAAAGCTTAGCCATCGCCAG-3′	this study
CreB5-R	5′-ACCTCTAGATGGGCAACGGGAG-3′	this study
CreC3-F	5′-CCATCTAGACAACGTGCCCGAC-3′	this study
CreC3-R	5′-TCAGGAGCTCCAGCAGCAGCAG-3′	this study

AmpG and AmpN not just AmpG in *P. aeruginosa*;[4] (ii) although two *ampD* homologues are found in the *S. maltophilia* genome, only $ampD_I$ is relevant to β-lactamase expression;[5] and (iii) the AmpR-AmpN-AmpG-$AmpD_I$ network is simultaneously involved in the expression of L1 and L2 β-lactamases, even though only the L2 gene, and not the L1 gene, is linked to an *ampR* gene.[6,7] The involvement of putative *S. maltophilia* PBPs in β-lactamase expression and β-lactam resistance have not been reported to date. In this article we focus on the relatedness of the putative PBP1a gene, *mrcA*, on L1/L2 expression and β-lactam resistance and involvement of the AmpR-AmpN-AmpG-$AmpD_I$ regulon and CreBC(BlrAB) two-component regulatory system in this.

Materials and methods

Bacterial strains, plasmids and primers

A complete list of bacterial strains, plasmids and primers used in this study is summarized in Table 1. All PCR primers used in this study were designed based on the *S. maltophilia* K279a genome sequence.[8]

Mutant construction

Disruption of *ampR*, $ampD_I$ and *ampNG* was performed as described previously.[4] To disrupt *mrcA*, a 1584 bp DNA fragment containing the partial *mrcA* gene was obtained by PCR amplification using primers MrcA-F/MrcA-R (Table 1) and cloned into vector pEX18Tc[4] to yield plasmid

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pMrcA1584. This plasmid was digested with PstI to delete a 207 bp fragment internal to the *mrcA* gene, generating plasmid pΔmrcA upon re-ligation of the cut DNA.

To disrupt the *creBC* operon, two non-overlapping DNA fragments corresponding to sequences upstream and downstream of the operon were obtained by PCR using primers CreB5-F/CreB5-R and CreC3-F/CreC3-R (Table 1) and the PCR amplicons were subsequently ligated together into the pEX18Tc, giving rise to plasmid pΔBC. The disruption mutants were constructed in *S. maltophilia* using a sucrose selection suicide delivery system described previously.[4]

Determination of β-lactamase activity and antimicrobial susceptibility tests

Crude cell extracts for determination of β-lactamase activity were prepared as previously described.[4] MICs of antimicrobials were determined by a standard 2-fold serial agar dilution method according to the guidelines of the CLSI.[4] The MICs of cefepime, imipenem and meropenem were determined using Etest strips (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions.

Nucleotide sequence accession numbers

The nucleotide sequences of *S. maltophilia* KJ partial *mrcA* gene have been deposited in GenBank under accession no. HM053614.

Results

Analysis of the mrcA gene of S. maltophilia

The *S. maltophilia* genome sequence strain K279a is genetically closely related to *S. maltophilia* KJ based on our previous studies[4,5,7,9] and was used as a reference strain for preliminary survey of putative PBP genes. The *mrcA* gene is identified as Smlt3826 in the *S. maltophilia* K279a genome. This gene is predicted to encode an 807 amino acid protein, which has 41%, 39%, 33%, 32% and 32% identity to *E. coli* PBP1a, 1b and 1c and *S. maltophilia* putative PBP1b (Smlt3681) and PBP1c (Smlt3602), respectively.

The deduced amino acid sequences encoded by the 1584 bp partial *mrcA* gene amplified by PCR from *S. maltophilia* strain KJ corresponded to amino acids 36–559 of *S. maltophilia* K279a PBP1a (Smlt3826), with a protein identity of 99%. Compared with wild-type KJ, the KJΔmrcA mutant displayed an ~100-fold increase in basal β-lactamase activity in the absence of the β-lactam treatment. The enzyme was still slightly inducible (~2-fold) upon the challenge with cefuroxime (Table 2), but the cefuroxime-induced β-lactamase activity seen in KJΔmrcA was nearly identical to that in wild-type KJ (Table 2). Furthermore, even though inactivation of *mrcA* raises the basal level of β-lactamase activity, it does not appear to affect β-lactam MICs according to the CLSI standard method used.

Basal β-lactamase activity of KJΔmrcA includes L1 and L2

L1 and L2 are normally co-induced by β-lactam challenge. To see whether both β-lactamases are overproduced upon disruption of *mrcA*, the Δ*mrcA* allele was introduced into KJΔL1 (an L1 isogenic mutant) and KJΔL2 (an L2 isogenic mutant).[9] In the absence of β-lactams, mutants KJΔL1ΔmrcA and KJΔL2ΔmrcA exhibited a detectable β-lactamase activity of 716±92 U/mg and 437±51 U/mg, respectively, indicating that loss of *mrcA* causes L1 and L2 to be coordinately up-regulated.

Relationship between mrcA and known components of the S. maltophilia β-lactamase induction network

To understand whether the effect on β-lactamase production seen upon loss of *mrcA* is dependent upon known β-lactamase regulatory proteins, AmpR, AmpN-AmpG and AmpDI, Δ*ampR*, Δ*ampNG* and $\Delta ampD_I$ alleles were introduced into mutant KJΔmrcA. In the mutants KJΔRΔmrcA and KJΔNGΔmrcA,

Table 2. β-Lactamase activities and β-lactam MICs of *S. maltophilia* KJ and its derived mutants

	β-Lactamase activity (U[a]/mg)					MIC (mg/L)				
S. maltophilia	basal	induced[b]	PIP	CAR	CXM	FOX	FEP	IPM	MEM	ATM
KJ	10±1.8	1868±215	1024	1024	2048	1024	64	>32	>32	>2048
KJΔmrcA	1012±190	2496±275	2048	2048	2048	1024	64	>32	>32	>2048
KJΔNG	5±0.8	6±0.7	16	2	32	32	2.4	2	0.38	16
KJΔNGΔmrcA	12±2.1	14±1.2	16	2	32	32	2.4	2	0.38	16
KJΔDI	4448±752	4392±460	1024	1024	2048	1024	96	>32	>32	>2048
KJΔDIΔmrcA	4751±876	6040±823	2048	2048	2048	1024	96	>32	>32	>2048
KJΔR	10±0.9	8±0.9	16	2	32	32	0.64	12	0.98	16
KJΔRΔmrcA	15±1.1	11±1.4	16	2	32	32	0.64	12	0.98	16
KJΔDIΔBC	4026±521	4145±614	1024	1024	2048	1024	96	>32	>32	>2048
KJΔmrcAΔBC	936±101	1871±321	2048	2048	2048	1024	64	>32	>32	>2048
KJΔDIΔmrcAΔBC	4554±976	5740±923	2048	2048	2048	1024	96	>32	>32	>2048

PIP, piperacillin; CAR, carbenicillin; CXM, cefuroxime; FOX, cefoxitin; FEP, cefepime; IPM, imipenem; MEM, meropenem; ATM, aztreonam.
[a]One unit of β-lactamase activity is defined as 1 nmol of nitrocefin hydrolysed per minute. Results are expressed as the mean±SD of three independent determinations.
[b]50 mg/L cefuroxime as the inducer.

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β-lactamase production returned to basal compared with KJΔmrcA, and β-lactam MICs decreased (Table 2), indicating that the Δ*mrcA*-derived basal-level β-lactamase derepression depends on functional AmpN-AmpG permease and AmpR. In addition to KJΔmrcA, mutation of *ampD*$_I$ has been reported to cause basal β-lactamase hyperproduction in *S. maltophilia*.[5] Accordingly, it was of interest to test whether Δ*mrcA* and Δ*ampDI* have a synergic effect on β-lactamase expression and β-lactam MICs. Table 2 shows that the β-lactamase activity of KJΔDIΔmrcA was very similar to that of KJΔDI, but about 4-fold higher than that of KJΔmrcA. In addition, β-lactam MICs for KJΔmrcA, KJΔDI and KJΔDIΔmrcA were almost identical.

No evidence that the CreBC two-component regulatory system is involved in β-lactamase expression and β-lactam susceptibilities of KJΔDI, KJΔmrcA and KJΔDIΔmrcA

The finding that the BlrAB regulatory system controls the expression of β-lactamases in *Aeromonas jandaei*[10] and that a homologous system (CreBC) is important for β-lactam resistance in *P. aeruginosa* PBP4 loss of function mutants[3] prompted us to test the involvement of the *blrAB*(*creBC)* homologue in β-lactamase production and β-lactam resistance in *S. maltophilia* KJΔDI, KJΔmrcA and KJΔDIΔmrcA.

CreB(BlrA) and CreC(BlrB) of *S. maltophilia* K279a were identified as Smlt1436 and Smlt1437, with identities of 50% and 47% with CreB and CreC of *P. aeruginosa*, respectively. A Δ*creBC* allele was introduced into strains KJΔDI, KJΔmrcA and KJΔDIΔmrcA. Table 2 shows that inactivation of *creBC* did not affect the basal or cefuroxime-induced β-lactamase activities or the MICs of β-lactams of these strains, indicating that *creBC* is not involved in Δ*ampDI*-derived and Δ*mrcA*-derived β-lactamase hyperproduction in *S. maltophilia*. Using the CLSI standard method, β-lactam MICs for *S. maltophilia* KJ did not increase upon disruption of *mrcA* or *ampD*$_I$, and there is no evidence that CreBC is involved in resistance, but this possibility cannot be totally excluded.

Discussion

The relationship between PBP activity and chromosomal β-lactamase production and β-lactam resistance has been extensively studied. PBP2 is required for the induction of the *C. freundii* β-lactamase.[11] Moreover, a strong β-lactamase inducer must inhibit all DD-carboxypeptidase PBPs (e.g. PBP4) as well as the essential PBPs 1a, 1b and 2.[12,13] Recently it was demonstrated that inactivation of the *dacB*-encoded PBP4 of *Aeromonas hydrophilia*[14] or *P. aeruginosa*[3] causes overproduction of β-lactamase and elevated MICs of β-lactams. In this study, KJΔmrcA exhibited a very high basal level of β-lactamase activity, to some extent being reminiscent of the *dacB* mutant of *P. aeruginosa* (PAΔ*dacB*).[3]

While KJΔmrcA and PAΔ*dacB* have a similar phenotype of basal-level β-lactamase hyperproduction, some distinctions between them were observed:

(i) PAΔ*dacB* increases the production of basal and induced AmpC compared with wild-type (PAO1).[3] However, KJΔmrcA only affects basal L1/L2 production and does not augment their induction.

(ii) PAΔ*dacB* exhibits higher β-lactam MICs than PAO1,[3] but inactivation of *mrcA* does not further increase the already very high MICs of β-lactams for *S. maltophilia* KJ.

(iii) Simultaneous inactivation of *dacB* and *ampD* produces a synergistic effect on the β-lactamase expression and β-lactam MICs in *P. aeruginosa*.[3] However, no such synergy was seen in *S. maltophilia*.

(iv) In *P. aeruginosa*, the CreBC two-component regulatory system is activated upon *dacB* inactivation and is involved in the Δ*dacB*-derived β-lactam resistance, but not Δ*dacB*-derived *ampC* overexpression.[3] While CreBC is not involved in Δ*mrcA*-derived and Δ*ampD*$_I$-derived β-lactamase hyperproduction, it is not certain from these data whether it has a role in β-lactam resistance in *S. maltophilia*. Of course, it cannot be ruled out that there is a two-component system other than the putative CreBC system identified here involved in Δ*mrcA*-derived β-lactamase expression and β-lactam resistance in *S. maltophilia*.

Alterations in PBPs, either in quantity or in sequence, is an important mechanism for β-lactam resistance in Gram-positive bacteria.[15] In general, alterations in PBPs of Gram-positive bacteria prevent β-lactam saturation, and so there is little effect on biological function and peptidoglycan synthesis. Nevertheless, in Gram-negative bacteria with the chromosomal *ampR*-β-lactamase genetic module, when a PBP loses its biological activity in peptidoglycan synthesis either by mutation[3] or by β-lactam saturation, this results in changes in peptidoglycan structure and disturbs the balance of the degraded peptidoglycan components in the cytosol, causing induction of the chromosomal β-lactamase gene, something that is essential for β-lactam resistance. Since every bacterium has its unique profile of PBPs, the impact of each PBP on chromosomal β-lactamase expression in different bacteria is likely to be diverse.

Funding

This work was supported by the National Science Council (NSC 98-2320-B-039-011-MY3) and the China Medical University (CMU99-S-42).

Transparency declarations

None to declare.

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