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, ampDI 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 ΔmrcA-derived basal β-lactamase hyperproduction was ampR and ampN-ampG dependent. Simultaneous inactivation of ampDI and mrcA did not augment β-lactamase production over and above that seen in an ampDI mutant alone. Furthermore, we could find no evidence for a role of the creBC two-component regulatory system in basal L1/L2 β-lactamase hyperproduction in a ΔampDI or ΔmrcA 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...
AmpG and AmpN not just AmpG in *P. aeruginosa*; (ii) although two ampD homologues are found in the *S. maltophilia* genome, only ampD3 is relevant to  β-lactamase expression; and (iii) the AmpR-AmpN-AmpG-AmpDI 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.

#### Mutant construction

Disruption of ampR, ampD3 and ampNG was performed as described previously. 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 to yield plasmid...
**Determination of β-lactamase activity and antimicrobial susceptibility tests**

Crude cell extracts for determination of β-lactamase activity were prepared as previously described. MICs of antimicrobials were determined by a standard 2-fold serial agar dilution method according to the guidelines of the CLSI. The MICs of cephalosporins, 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 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 KJ ΔmrcA 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 KJ ΔmrcA 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.

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

<table>
<thead>
<tr>
<th>S. maltophilia</th>
<th>β-Lactamase activity (U/mg)</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>basal</td>
<td>induced&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>KJ</td>
<td>10 ± 1.8</td>
<td>18687 ± 215</td>
</tr>
<tr>
<td>KJ ΔmrcA</td>
<td>1012 ± 190</td>
<td>2496 ± 275</td>
</tr>
<tr>
<td>KJ ΔNGmrcA</td>
<td>5 ± 0.8</td>
<td>67 ± 0.7</td>
</tr>
<tr>
<td>KJ ΔANGmrcA</td>
<td>12 ± 2.1</td>
<td>14 ± 1.2</td>
</tr>
<tr>
<td>KJ ΔDI</td>
<td>4448 ± 752</td>
<td>4392 ± 460</td>
</tr>
<tr>
<td>KJ ΔDI ΔmrcA</td>
<td>4751 ± 876</td>
<td>6040 ± 823</td>
</tr>
<tr>
<td>KJ ΔR</td>
<td>10 ± 0.9</td>
<td>8 ± 0.9</td>
</tr>
<tr>
<td>KJ ΔR ΔmrcA</td>
<td>15 ± 1.1</td>
<td>11 ± 1.4</td>
</tr>
<tr>
<td>KJ ΔDI ΔBC</td>
<td>4026 ± 521</td>
<td>4145 ± 614</td>
</tr>
<tr>
<td>KJ ΔmrcA ΔBC</td>
<td>936 ± 101</td>
<td>1871 ± 321</td>
</tr>
<tr>
<td>KJ ΔDI ΔmrcA ΔBC</td>
<td>4554 ± 976</td>
<td>5740 ± 923</td>
</tr>
</tbody>
</table>

P, piperacillin; CAR, carbenicillin; CXM, cefuroxime; FOX, cefoxitin; FEP, cefepime; IPM, imipenem; MEM, meropenem; ATM, aztreonam.

<sup>a</sup> 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.

<sup>b</sup> 50 mg/L cefuroxime as the inducer.
β-lactamase production returned to basal compared with KJΔmrcA, and β-lactam MICs decreased (Table 2), indicating that the ΔmrcA-derived basal-level β-lactamase derepression depends on functional AmpN-AmpG permease and AmpR. In addition to KJΔmrcA, mutation of ampD has been reported to cause basal β-lactamase hyperproduction in S. maltophilia.\(^5\) Accordingly, it was of interest to test whether ΔmrcA and ΔampD have a synergistic effect on β-lactamase expression and β-lactam MICs. Table 2 shows that the β-lactamase activity of KJΔΔmrcA was very similar to that of KJΔΔ, but about 4-fold higher than that of KJΔmrcA. In addition, β-lactam MICs for KJΔmrcA, KJΔΔ and KJΔΔΔmrcA were almost identical.

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

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* KJΔΔ, KJΔmrcA and KJΔΔΔmrcA.

CreB (BlrA) and CreC (BlrB) of *S. maltophilia* K279a were identified as Smrt11436 and Smrt11437, with identities of 50% and 47% with CreB and CreC of *P. aeruginosa*, respectively. A creBC allele was introduced into strains KJΔΔ, KJΔmrcA and KJΔΔΔmrcA. 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 ΔampD- or ΔmrcA-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, 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* \(^5\) 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* (PAΔdacB).\(^3\)

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

(i) PAΔdacB increases the production of basal and induced AmpC compared with wild-type (PAO1).\(^5\) However, KJΔmrcA only affects basal L1/L2 production and does not augment their induction.

(ii) PAΔdacB 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*.\(^5\) 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 ΔdacB-derived β-lactam resistance, but not ΔdacB-derived ampC overexpression.\(^3\) While CreBC is not involved in ΔmrcA-derived and ΔampD-derived β-lactamase hyperproduction, it is not certain that 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 ΔmrcA-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\(^2\) 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 every bacterium has its unique profile of PBPs, the impact of

**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.

**References**


