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SCIENCE DIRECT
Gene 358 (2005) 82 – 92
GENE
SECTION
FUNCTIONAL GENOMICS
www.elsevier.com/locate/gene

# Characterization of *poxB*, a chromosomal-encoded *Pseudomonas aeruginosa* oxacillinase

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Received 2 November 2004; received in revised form 11 April 2005; accepted 17 May 2005
Available online 24 August 2005
Received by D.L. Court

**Abstract**

*Pseudomonas aeruginosa* is the major pathogen associated with morbidity and mortality of patients with cystic fibrosis. One of the reasons for the failure of β-lactam antibiotic regimens appears to be mediated by de-regulation of the *ampC* gene, encoding the chromosomal Ambler's Class C β-lactamase. Currently, the AmpC is the only known chromosomal β-lactamase whose expression is regulated by a transcriptional regulator, AmpR. We generated an *ampC* mutation in the prototypic *P. aeruginosa* strain PAO1. The mutation in *ampC* did not abolish the β-lactamase activity entirely suggesting the expression of yet another unreported β-lactamase. Our genomic analysis revealed the presence of an open reading frame encoding a protein with high homology to the Class D β-lactamases, commonly known as oxacillinases. The gene was named *poxB* for *Pseudomonas ox*acillinase. Cloning and expression of *poxB* in *Escherichia coli* conferred β-lactam resistance to the host. We detected the presence of *poxB* both in clinical and environmental isolates. Our studies show that *P. aeruginosa* possesses two β-lactamases, AmpC and PoxB, which contribute to its resistance against a wide spectrum of β-lactam antibiotics.


*Keywords:* Antibiotic resistance; *ampc*; Chromosomal Class D β-lactamase

## 1. Introduction

*Pseudomonas aeruginosa* is a dominant pathogen in patients with cystic fibrosis (CF), chronic obstructive pulmonary disease, severe burns and patients in intensive care units. The presence of this organism is associated with a high risk of morbidity and mortality in CF. The current treatment against *P. aeruginosa* includes a combination of β-lactam antibiotics and aminoglycosides (Frederiksen et al., 1996). However, resistance to β-lactam antibiotics is common. *P. aeruginosa* employs several mechanisms to neutralize β-lactams including multidrug efflux pumps, restricted membrane permeability, alteration in the penicillin-binding proteins and expression of β-lactam hydrolyzing enzymes β-lactamases (Lambert, 2002). However, resistance to β-lactam antibiotics due to the expression of β-lactamases appears to be by far the most prominent mechanism in CF strains.

According to the Nomenclature Committee of the International Union of Biochemistry, β-lactamases are a group of diverse enzymes that hydrolyze amides, amidines and other C–N bonds. Owing to the complexity of these enzymes, four major classification schemes were proposed by Richmond and Sykes, Ambler, Mitsuhashi and Inoue and Bush et al. (Bush et al., 1995). Among these, the Ambler system has been widely adopted. Ambler's scheme differentiates β-lactamases by primary amino acid sequences into four classes, Classes A, B, C and D (Ambler, 1980). All four classes have been documented in *P. aeruginosa*.

*Abbreviations:* bp, base pair(s); CF, Cystic Fibrosis; *E*, *Escherichia*; kb, kilobase(s); Gm, Gentamycin; ORF, open reading frame; *P*, *Pseudomonas*; PCR, Polymerase Chain Reaction.

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doi:10.1016/j.gene.2005.05.027

Ambler molecular class A was initially described in Gram-positive plasmids. However, plasmid-, transposon- and chromosomally mediated β-lactamases (VHS, PER, TEM and SHV) in Gram-negative bacteria are not uncommon (Couture et al., 1992). This class of enzymes, dubbed penicillinases, exhibits the highest degree of sequence variability and kinetic properties. Plasmid-encoded Ambler class A β-lactamases identified in *P. aeruginosa* are active against carbenicillin and are hence referred to as CARB or *Pseudomonas*-specific β-lactamases (Lachapelle et al., 1991). In addition, VHS-, PER- and TEM-derived β-lactamases have also been reported in *P. aeruginosa* (Weldhagen et al., 2003).

The Ambler Class B contains a small number of $Zn^{2+}$ metallo-β-lactamases, whereby their activities could be inhibited by EDTA. IMP-1 is the first metallo-β-lactamase described in *P. aeruginosa* (Watanabe et al., 1991). Its gene, $bla_{IMP}$, appears to be dispersed among *P. aeruginosa* and other gram-negative rods in Japan (Senda et al., 1996). An integron-borne metallo-β-lactamase gene, $bla_{VIM}$, which was originally described in *P. aeruginosa* isolated in Italy, gives rise to the resistance of meropenem and imipenem (Tsakris et al., 2000).

The Ambler Class C enzymes are active against cephalosporins, hence they are known as cephalosporinases. They are chromosome-encoded and synthesized by most Gram-negative bacteria. The known sequences of these enzymes are highly conserved. The *P. aeruginosa* Class C cephalosporin-hydrolyzing chromosomal β-lactamase is encoded by *ampC* (Lodge et al., 1990). The inducible expression of AmpC is regulated by an upstream divergently transcribed gene, *ampR* that encodes a transcriptional regulator (Lodge et al., 1990).

Due to the structural similarity between Class A and Class D enzymes, Couture et al. suggested the use of 26 conserved amino acid residues as the class D standard numbering scheme (DBL numeration) (Couture et al., 1992). This group of β-lactamases is called oxacillinases due to their ability to degrade isoxazolyl β-lactams such as oxacillin and methicillin (Dale and Smith, 1972). Clavulanic acid, on the other hand, serves as a good inhibitor for these enzymes (Medeiros et al., 1985). Plasmid- and transposon-mediated oxacillin-hydrolyzing β-lactamases in *P. aeruginosa* are common yet complex. A nosocomial outbreak with a *P. aeruginosa* extended-spectrum of Class D β-lactamases (ESBLs) has been reported (Poirel et al., 2002) and more episodes are expected to arise in the near future.

The Ambler Class C enzyme, *ampC*, has been cloned and sequenced from the prototypic *P. aeruginosa* PAO1 (Lodge et al., 1990). Here we report the characterization of the *ampC* mutant that led to the discovery of a second chromosomally encoded β-lactamase belonging to Class D oxacillinases. The identified open reading frame (ORF) PA5514 is named *poxB* for *P. aeruginosa* *ox*acillinase.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids and media

*Escherichia coli* used in this study were DH5α ($F^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17*($r_k^-$, $m_k^+$) *phoA supE44 λ-thi-1 gyrA96 relA1;* New England Biolabs) and TOP10F' (F' {$lacI^q$, Tn10($Tet^R$)} *mcrA* Δ*(mrr-hsdRMS-mcrBC)* ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139 D(ara-leu)7697 galU galK rpsL* ($Str^R$) *endA1 nupG*; Invitrogen). *P. aeruginosa* strains and plasmids constructed in this study were shown in Table 1. *Escherichia coli* and *P. aeruginosa* were routinely cultured in Luria-Bertani medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, per liter). *Pseudomonas* Isolation Agar (Difco) was used in triparental mating experiments. Antibiotics were used at the following concentrations (per milliliter unless indicated otherwise): ampicillin at 50 μg, tetracycline at 20 μg, gentamycin at 15 μg for *E. coli* and carbenicillin at 300 μg, gentamycin at 300 μg and tetracycline at 60 μg for *P. aeruginosa*. For induction, 25 μg and 500 μg of benzylpenicillin were used for *E. coli* and *P. aeruginosa*, respectively.

### 2.2. DNA manipulations

All molecular techniques were performed according to standard protocols (Ausubel et al., 1999).

### 2.3. Insertional inactivation of ampC gene

A 2150-bp *ampC* fragment was amplified by polymerase chain reaction (PCR) using SBJ05*ampC*For (5′-G*GAATTC*-TGAGGCCGCGCGGCAGACGCTTGAACA-3′) and SBJ06*ampC*Rev (5′-CG*GGATCC*AACCCCGGCGCGGT-GGCCAGTCCCGCCAA-3′) primers with flanking *Eco*RI and *Bam*HI sites (the italicized portion in the sequence of the primers), respectively (Fig. 1). The PCR product was ligated to pCRII-TOPO vector (Invitrogen, CA), generating pSJ02. A 899-bp *Sal*I fragment containing the gentamycin cassette, *aacCI*, was retrieved from pUCGm (Accession No: U04610) and inserted into the *Sal*I site of *ampC*. This disrupted the reading frame of *ampC* in pSJ02. Then, a blunt-ended 3049-bp *Eco*RI fragment containing the *ampC*::*aacCI* was ligated to the *Sma*I-cut suicide vector, pEX100T (Accession No: U17500), to yield pSJ08. This plasmid was conjugated into *P. aeruginosa* PAO1 with a helper strain harboring pRK2013 ($Km^R$; ColE1*ori*-Tra $(RK2)^+$: Figurski and Helinski, 1979). The merodiploids resulting from homologous recombination were selected with PIA containing gentamycin. $Gm^R$ colonies were then screened for gentamycin resistance and carbenicillin sensitivity by replica plating for the loss of plasmid. The insertion was confirmed by PCR and restriction analysis on the PCR product (data not shown). The PAO1 isogenic