Cloning and functional characterization of the ambler Class C β-lactamase of Yersinia ruckeri

Hedi Mammeri, Laurent Poirel, Hasan Nazik & Patrice Nordmann

Service de Bactériologie-Virologie, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Paris-Sud, Le Kremlin-Bicêtre, France

Correspondence: Patrice Nordmann, Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre cedex, France. Tel.: +33 1 45 21 36 32; fax: +33 1 45 21 63 40; e-mail: nordmann.patrice@bct.ap-hop-paris.fr

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Abstract

Yersinia ruckeri is a gram-negative pathogen causing enteric redmouth disease in salmonids. Previous studies have reported that Y. ruckeri harbors an ampC gene that is expressed at low level. In this present work, the entire ampC gene of Y. ruckeri was cloned and expressed in Escherichia coli. The AmpC enzyme confers resistance to aminopenicillins and narrow-spectrum cephalosporins, which fit well with the kinetic properties of the purified enzyme. Phylogenetic analysis showed that YRC-1 did not share significant sequence identity with known plasmid-mediated or chromosomal AmpC enzymes. This work provides further evidence that fish-pathogenic gram-negative rod species may constitute a reservoir of antibiotic resistance genes.

Introduction

Yersinia ruckeri is a gram-negative rod which is a water saprophyte firstly described in 1955. This bacterium is the causative agent of enteric redmouth disease (ERM), a serious infection disease of the farm fish (Furones et al., 1993). In a previous study, strains of Y. ruckeri were shown to be naturally sensitive to a variety of β-lactam antibiotics, including amoxicillin and cephalosporins, indicating the absence of expressed β-lactamase or a low-level expression of these enzymes (Stock et al., 2002).

Recently, Schiefer et al. (2005) have shown that Y. ruckeri harbored an ampC gene which is expressed constitutively at low level. A partial sequence of the gene (GenBank accession no. AF525881) was amplified using a PCR-based strategy. It contained only 685 nucleotides, which did not allow complete phylogenetic analysis of the gene. Moreover, the hydrolysis spectrum and the kinetic parameters of the enzyme were not determined.

In the present work, we have cloned, sequenced, and analyzed the complete ampC gene of Y. ruckeri 1006/94 isolate, in order to determine precisely its genetic relatedness with either known chromosomal or plasmid-mediated cephalosporinas. In addition, determination of the enzymatic constants of the purified β-lactamase was carried out.

Materials and methods

Bacterial strains and plasmids

Yersinia ruckeri 1006/94 isolate was kindly provided by the National Veterinary institute (Oslo, Norway). Escherichia coli DH10B and E. coli J53 (AzideR) were used for transformation and conjugation experiments, respectively.

Cloning of β-lactamase gene

Whole-cell DNA of Y. ruckeri isolates 1006/94 was extracted and digested by HindIII as described previously (Mammeri et al., 2004). DNA fragments were ligated into HindIII-restricted phagemid vector pBK-CMV (Stratagene, Amsterdam, The Netherlands).

Recombinant phagemids were transformed into E. coli strain DH10B by electroporation with a Gene Pulser II apparatus (Bio-Rad, Ivy-sur-Seine, France). Transformants were selected on Trypticase soy agar containing ampicillin (100 µg mL⁻¹) and kanamycin (30 µg mL⁻¹). Recombinant plasmids were purified with a plasmid Midi kit (QIAGEN, Courtaboeuf, France). The cloned β-lactamase genes were sequenced on both strands by using an Applied Biosystems sequencer (ABI 377). The nucleotide and deduced protein sequences were analyzed with software available over the Internet from the National Center for Biotechnology.
Information website (www.ncbi.nlm.nih.gov). The ClustalW program (www.infobiogen.fr) was used for the alignment of multiple protein sequences. The prediction of the leader peptide cleavage site was performed at the following website: http://www.cbs.dtu.dk/services/SignalP/.

Conjugation and transformation experiments

Conjugation and transformation experiments were performed as described previously (Sambrook & Russell, 2001). Plasmid-DNA of Y. ruckeri 1006/94 was extracted using the Qiagen plasmid DNA midi kit (Qiagen, Paris, France). Azide (100 μg mL⁻¹) combined with amoxicillin (100 μg mL⁻¹), or amoxicillin (100 μg mL⁻¹) alone, were used as selective agents for conjugative and transformation experiments, respectively.

Isoelectric focusing (IEF) analysis

The β-lactamase extracts from cultures of Y. ruckeri isolate and purified enzymes were subjected to analytical IEF analysis as previously described (Mammeri et al., 2004). The focused β-lactamases were detected by overlaying the gel with a 1 mM nitrocefin solution (Calbiochem, Merck Eurolab SAS, Fontenay-sous-Bois, France).

Antimicrobial agents and minimal inhibitory concentration (MIC) determination

MICs were determined by an agar dilution technique on Mueller-Hinton agar (Sanofi-Diagnostics Pasteur, Paris, France) with an inoculum of 10⁴ CFU per spot and were interpreted according to the guidelines of the CLSI (Clinical and Laboratory Standards Institute, 2005).

β-Lactamase purification

Recombinant E. coli DH10B (pBK-1006/94) strain was grown overnight at 37°C in 4 L of Trypticase soy broth containing amoxicillin (100 μg mL⁻¹) and kanamycin (30 μg mL⁻¹), resuspended in 40 mL of 100 mM phosphate buffer (pH 7), disrupted by sonication, and centrifuged at 20,000 g for 1 h at 4°C as previously described (Mammeri et al., 2004). β-Lactamase extracts were filtered through a 0.45 μm pore size filter (Millipore, Saint-Quentin-en-Yvelines, France), dialyzed overnight at 4°C against 20 mM Tris (pH 7.5), and loaded onto a pre-equilibrated Q-Sepharose column (Amersham Pharmacia Biotech, Orsay, France). The flowthrough fractions containing the β-lactamase were recovered and dialyzed against 50 mM phosphate buffer (pH 6) before being loaded onto a pre-equilibrated S-Sepharose column (Amersham Pharmacia Biotech). The enzyme was eluted by a linear NaCl gradient (0 to 1 M) in the same buffer. The eluted fractions with the highest β-lactamase activity (nitrocefin test) were pooled and dialyzed against 100 mM phosphate buffer (pH 7). To assess the purity of the extract, purified enzyme was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Mammeri et al., 2004).

Kinetic measurements

Purified β-lactamase AmpC YRC-1 was used for kinetic measurements (Km and kcat), which were realized at 30°C in 100 mM sodium phosphate (pH 7.0). The rates of hydrolysis were determined with a Pharmacia ULTROSPEC 2000 spectrophotometer and were analyzed by using the SWIFT II software (Amersham Pharmacia Biotech). Km and kcat values were determined by analyzing β-lactam hydrolysis under initial rate conditions by using the Eadie–Hofstee linearization of the Michaelis–Menten equation as previously described (Cornish-Bowden, 1995). When the Km value was very low, the Ki value was determined from the initial rates at saturating substrate concentrations ([S] >> Km).

Southern analysis and I-CeuI techniques

Southern hybridization experiments (Sambrook & Russell, 2001) were performed using 0.8% electrophoresis gel containing whole-cell DNA of Y. ruckeri 1006/94 transferred onto a nylon membrane that was then hybridized with PCR-obtained internal fragments for blav_YRC_1–1 (IntA, 5'–TGAATTTGCAACCTATACC–3'; IntB, 5'–AATGAAAGC–3'). Visualization of hybridization was performed using the ECL nonradioactive hybridization kit as described by the manufacturer (Amersham Pharmacia Biotech). Additionally, chromosomal locations of the β-lactamase genes were investigated using the I-CeuI technique (Liu et al., 1993). Whole-cell DNA of the Y. ruckeri 1006/94 was digested with endonuclease I-CeuI (New England Biolabs, Ozyme, Saint-Quentin-en-Yvelines, France), which digests a 26 bp sequence in rrn genes for the 23S large subunit rRNA. After digestion, separation of the resulting fragments was performed on a CHEF-DRII apparatus used for pulsed-field gel electrophoresis (PFGE), as described previously (Liu et al., 1993). The sizes of the I-CeuI-generated fragments were determined by comparison with those of Lambda-ladder molecular weight marker (Bio-Rad). A Southern transfer of the PFGE gel was hybridized with specific probes for blav_YRC_1–1 and a probe for 16rRNA genes made of PCR-generated fragments, using universal primers 8–24 (5'–AGAGTTTGATCCTGCTGAGA–3' and 1512–1491 (5'–ACGAGTACCTTTGTACGACTT–3').

Nucleotide sequence accession number

The nucleotide sequence of the blav_YRC_1–1 gene from Y. ruckeri isolate 1006/94 has been submitted to the GenBank...
nucleotide database under the accession numbers no. DQ185144.

Results and discussion

Cloning of β-lactam resistance markers of Yersinia ruckeri 1006/94 followed by its expression in Escherichia coli gave recombinant plasmid pBK-1006/94. This plasmid gave a resistance phenotype consistent with AmpC β-lactamase-type. The MICs of β-lactams of Y. ruckeri 1006/94 corresponded with that of an AmpC-type enzyme expressed at a low level (Table 1). Escherichia coli DH10B (pBK-1006/94) expressed a similar resistant profile but an higher level of resistance. In all cases, susceptibility to extended-spectrum cephalosporins, and imipenem, was observed. The inducibility of resistance to β-lactams, as evidenced on a routine antibiotic by an antagonism line between cefoxitin or imipenem and cefotaxime discs was not observed for Y. ruckeri 1006/94 or corresponding E. coli recombinant strain (data not shown).

DNA sequence analysis of the 1.6 kb insert of pBK-1006/94 revealed an open reading frame (ORF) of 1152 bp that shared significant sequence identity with the Ambler class C β-lactamases genes. Within the deduced amino acid sequence of the mature protein, an SXXK tetrad, characteristic of β-lactamases possessing a serine active site, was found at positions 150–152 (Mammeri, 2004). Three structural elements characteristic of AmpC β-lactamases were also found (Mammeri, 2004): YSN at positions 64–69 (Mammeri, 2004). Three structural elements characteristic of AmpC β-lactamases were also found (Mammeri, 2004): YSN at positions 64–69 (Mammeri, 2004). Three structural elements characteristic of AmpC β-lactamases were also found (Mammeri, 2004): YSN at positions 64–69 (Mammeri, 2004). Three structural elements characteristic of AmpC β-lactamases were also found (Mammeri, 2004): YSN at positions 64–69 (Mammeri, 2004).

Table 1. Minimum inhibitory concentrations of β-lactams for Yersinia ruckeri 1006/94 isolate, recombinant Escherichia coli DH10B (pBK-1006/94), and E. coli DH10B

<table>
<thead>
<tr>
<th>β-Lactam(s)</th>
<th>Y. ruckeri 1006/94</th>
<th>E. coli DH10B (pBK-1006/94) producing YRC-1</th>
<th>E. coli DH10B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>4</td>
<td>&gt; 512</td>
<td>2</td>
</tr>
<tr>
<td>Amoxicillin – CLA</td>
<td>4</td>
<td>&gt; 512</td>
<td>2</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ticarcillin – CLA</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Piperacillin – TZB</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>64</td>
<td>&gt; 512</td>
<td>4</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>4</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>4</td>
<td>256</td>
<td>0.5</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>&lt; 0.06</td>
<td>0.125</td>
<td>&lt; 0.06</td>
</tr>
<tr>
<td>Cefetzidime</td>
<td>&lt; 0.06</td>
<td>1</td>
<td>&lt; 0.06</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>&lt; 0.06</td>
<td>0.25</td>
<td>0.06</td>
</tr>
<tr>
<td>Cefepime</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
</tr>
<tr>
<td>Cefpirome</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
</tr>
<tr>
<td>Imipenem</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
</tr>
</tbody>
</table>

CLA, clavulanic acid at 2 μg mL⁻¹; TZB, tazobactam at 4 μg mL⁻¹.

β-lactamase-type enzyme produced by Y. ruckeri 1006/94 was designated YRC-1 (for Y. ruckeri cephalosporinase). AmpC YRC-1 did not share sufficient sequence identity with known plasmid-mediated enzymes to be proposed as a progenitor. It was 74% and 73% identical to the chromosomal β-lactamases of Enterobacter cloacae (Coneicau et al., 2004) and Enterobacter aerogenes (Preston et al., 2000), respectively, which were the closest AmpC enzymes (Fig. 1). Yersinia species constitute an heterogeneous group according to their β-lactamase content. Yersinia frederiksenii and Yersinia enterocolitica express both AmpC and class A β-lactamases, whereas Yersinia bercovieri, Yersinia aldovae, and Y. ruckeri express only an AmpC β-lactamase (Schifer et al., 2005). The inducibility of the cephalosporinase expression is detectable in Y. bercovieri and Y. enterocolitica strains, whereas it was variable among Y. frederiksenii strains and was absent in all Y. aldovae and Y. ruckeri strains (Schifer et al., 2005). Moreover, sequence analysis of the AmpC β-lactamases from Y. aldovae and Y. ruckeri revealed only 66% identity, which showed the weak relatedness between these two enzymes. Yersinia ruckeri is sometimes referred to as Y. enterocolitica-like bacteria, even though it is a distinct species (Sulakvelidze, 2000). However, the question of whether it really belongs to the genus Yersinia is still a matter of controversy. Studies of DNA relatedness and biochemical reactions revealed that the strains were 30% related to both Serratia and Yersinia species, but that they were more similar to Yersinia by their biochemical reactions and their G+C content. Moreover, Y. ruckeri strains have been shown not to carry the inv-homologous gene sequences found in other Yersiniae (Kwaaga et al., 1992). Our data provide additional arguments that support the taxonomic position of Y. ruckeri distinct from the genus Yersinia.

Sequence analysis of the 380 bp laying upstream the ampC gene failed to detect any ampR gene encoding a putative LysR-type regulator known to be often associated to ampC genes. This observation was in accordance with the absence of inducibility of resistance on routine antibiotic. Conjugation and transformation experiments using plasmid DNA of Y. ruckeri 1006/94 failed to give AmpC-producing transconjugant or transformant, suggesting a chromosomal location of the ampC gene. Using the I-CelI technique, six DNA fragments were generated (Fig. 2a). All of the fragments hybridized with an rRNA probe (Fig. 2b). The blaYRC-1 probes hybridized with a c. 900 kb fragment of Y. ruckeri 1006/94 that gave also a positive signal with the rRNA probe (Fig. 2c), demonstrating a chromosomal location of these β-lactamase genes.

IEF analysis of a crude β-lactamase extract of isolate 1006/94 gave a single band with pI value of 8.3 that co-migrated with a β-lactamase extract obtained from E. coli DH10B (pBK-1006/94).
AmpC enzyme was purified to near homogeneity (> 99%), as deduced from SDS-PAGE analysis (data not shown). The specific activities, as determined with 100 μM cephalothin as the substrate, was 40 μmol min⁻¹ mg protein⁻¹. The catalytic efficiency of the purified β-lactamase AmpC YRC-1 was high against narrow spectrum cephalosporins, such as cephalothin, cefoxitin, cefuroxime, low against ceftazidime, and undetectable against ticarcillin, piperacillin, and imipenem. The novel extended-spectrum cephalosporins, such as cefepime and cefpirome, were only weakly hydrolyzed (Table 2). These kinetic parameters were very similar to those of other cephalosporinases (Fosse et al., 2003).

Although ERM can be controlled by vaccination, antimicrobial agents are still frequently used for the treatment of ERM outbreaks in fish farms (Gibello et al., 1999). It mainly relies on quinolones, such as oxolinic acid or flumequine, but it does not require β-lactams. Nevertheless, identification and characterization of resistant determinants, including β-lactamase genes, among water saprophytes are still of interest since recent studies have revealed the submarine origin of some plasmid-mediated-resistant markers. For examples, the blaFOX – 1 gene, which codes for a cephalosporinase, derived from the chromosomal ampC gene of Aeromonas caviae (Fosse et al., 2003), the blaOXA – 48 gene, which codes for a carbapenem-hydrolyzing oxacillinase, derived from the chromosomal blaOXA – 54 of Shewanella oneidensis (Poirel et al., 2004), and Shewanella algae is the reservoir of the plasmid-mediated quinolone resistance determinant qnrA (Poirel et al., 2005). This work provides further evidence that fish-pathogenic Enterobacteriaceae may constitute a reservoir of antibiotic resistance genes.

Fig. 1. Dendrogram obtained by parsimony analysis for representative natural AmpC β-lactamases. The origins of the cephalosporinases were as follows: AmpC Pseudomonas aeruginosa PAO1 (GenBank accession no. AE004827), AmpC Yersinia enterocolitica IP97 (GenBank accession no. X63149), AmpC Psychrobacter immobile (GenBank accession no. X83586), AmpC Escherichia coli K-12 (GenBank accession no. J01611), AmpC Serratia marcescens S3 (GenBank accession no. AF211348), AmpC Citrobacter freundii H224 (GenBank accession no. Y15129), AmpC Enterobacter aerogenes 97B (GenBank accession no. AF327324), AmpC Morganella morganii SLMO1 (GenBank accession no. Y10283), AmpC Enterobacter aerogenes 978 (GenBank accession no. AF211348), AmpC Citrobacter freundii H224 (GenBank accession no. Y15129), AmpC Enterobacter cloacae FFUL2En (GenBank accession no. M37839), AmpC Enterobacter cloacae P99 (GenBank accession no. X07274), AmpC Yersinia ruckeri 1006/94 (GenBank accession no. 185144), AmpC Aeromonas hydrophila T4291 (GenBank accession no. AJ276030), AmpC Psychrobacter immobile SL07 (GenBank accession no. AJ069931), AmpC Psychrobacter anthropi SL07 (GenBank accession no. AJ001618), AmpC Acinetobacter baumannii RYC 52763/97 (GenBank accession no. J01611). The alignment used for tree calculation was performed with ClustalW followed by minor adjustments. Percent amino acid identities to AmpC YRC-1 are indicated in parentheses.
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References


