Chromosomal Integration of a Cephalosporinase Gene from *Acinetobacter baumannii* into *Oligella urethralis* as a Source of Acquired Resistance to β-Lactams

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Clinical *Oligella urethralis* isolate COH-1, which was uncommonly resistant to penicillins and narrow-spectrum cephalosporins, was recovered from a 55-year-old patient with a urinary tract infection. Shotgun cloning into *Escherichia coli* and expression experiments gave recombinant clones expressing either an AmpC β-lactamase-type phenotype of resistance or a carbencillin-hydrolyzing β-lactama-like phenotype of resistance. The AmpC β-lactamase identified (ABA-1), which had a pI value of 8.2, had 98% amino acid identity with a chromosomally encoded cephalosporinase of *Acinetobacter baumannii*. A 820-bp insertion sequence element, ISOour1, belonging to the IS6 family of insertion sequence elements, was identified immediately upstream of *bla*ABAl, providing a −35 promoter sequence and likely giving rise to a hybrid promoter region. The carbencillin-hydrolyzing β-lactamase identified (CARB-8), which had a pI value of 6.4, differed from CARB-5 by two amino acid substitutions. Hybridization of *Ceu*ABAl fragment I-restricted DNA fragments of *O. urethralis* COH-1 with *bla*ABAl, *bla*CARB-8, and 16S rRNA-specific probes indicated the chromosomal integration of the β-lactamase genes. PCR and hybridization experiments failed to detect *bla*CARB-8 and *bla*ABAl-like genes in three *O. urethralis* reference strains, indicating that the β-lactamase genes identified were the source of acquired resistance in *O. urethralis* COH-1. This is one of the few examples of the interspecies transfer and the chromosomal integration of a gene encoding a naturally occurring β-lactamase.

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**Oligella urethralis** is a coccobacillary, aerobic, gram-negative rod. The *Oligella* genus is distinct from the genera *Moraxella* and *Neisseria*, whereas it shares close genetic and phenotypic relationships with the genera *Alcaligenes*, *Bordetella*, and *Taylorella* (37). *O. urethralis* is a commensal organism of the genital and urethral tracts with a low grade of pathogenicity. It is occasionally identified as a source of urinary tract infections (16), mainly occurring in immunocompromised patients, and is rarely responsible for invasive infections such as septic arthritis (26), septicemia (34), and peritonitis (36).

Susceptibility testing studies report that *O. urethralis* is generally susceptible to β-lactams; in one case, however, a strain was found to produce a nonidentified β-lactamase that conferred resistance to penicillins (34).

This work reports on an analysis of the β-lactamase resistance mechanisms of an *O. urethralis* clinical isolate. Two β-lactamases genes encoding a carbencillin-hydrolyzing β-lactamase and a cephalosporinase were identified, with the latter sharing almost perfect amino acid identity with that of *Acinetobacter baumannii*. The chromosomal integration of this cephalosporinase gene in *O. urethralis* makes it one of the few examples of the chromosome-to-chromosome transfer of a β-lactamase gene among two different species and underlines the fact that cephalosporinase genes may be the source of acquired resistance not only when they are plasmid encoded but also when they are integrated into the chromosome.

**MATERIALS AND METHODS**

**Bacterial strains.** *O. urethralis* COH-1 was isolated in December 2001 from a 55-year-old patient with multiple sclerosis and a urinary tract infection who had been hospitalized in the neurological unit of the Albert Chenevier Hospital (Créteil, France). Bacterial identification was performed by using the API 32GN system (bioMérieux, Marcy l'Etoile, France) and was confirmed by 16S RNA sequencing (data not shown).

*O. urethralis* reference strains CPI102456, CPI116103, and CPI5133 were from the Institut Pasteur (Paris, France) strain collection. Strains *Escherichia coli* DH10B and *E. coli* XL1 Blue were used for cloning and conjugation experiments, respectively. Isolates *A. baumannii* ABAC1 and ABAC2 were recovered from urine samples of two patients admitted to the same neurological unit of Albert Chenevier Hospital during the same period.

**Plasmid DNA extraction, conjugation, and transformation experiments.** Plasmid DNAs from *O. urethralis* COH-1 and recombinant *E. coli* DH10B were extracted by using the plasmid Midi kit (Qiagen, Courtaboeuf, France) by the method of Kado and Liu (21). Conjugation assays in solid and liquid media and transformation experiments were performed as described previously (14).

**Cloning experiments.** Whole-cell DNA of *O. urethralis* COH-1 was extracted as described previously (3). All enzymes used in the cloning experiments were from Amersham Pharmacia Biotech (Orsay, France). Whole-cell DNA was partially digested with *Sac*I and the fragments were ligated into BamHI-restricted phageid vector pBS-CMV (Stratagene, Amsterdam, The Netherlands). Recombinant phagemids were transformed into *E. coli* DH10B (Stratagene) by electroporation with a Gene Pulser II apparatus (Bio-Rad, Ivry-sur-Seine, France). Transformants were selected on Mueller-Hinton agar containing ampicillin (30 μg/ml) and kanamycin (30 μg/ml). Both strands of the cloned DNA inserts of the recombinant plasmids were sequenced 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 (http://www.ncbi.nlm.nih.gov/BLAST/).
PCR experiments and sequencing. PCR experiments were performed for detection of bla\textsubscript{CARB-8} and bla\textsubscript{ABA-1} in \textit{O. urethralis} reference strains with a series of primers consisting of primers CARB-8A and CARB-8B or primers ampC-AB1 and ampC-AB2 (Table 1). Primers preABA-1 and preABA-2 were used to amplify the entire sequences of the \textit{ampC} genes of the \textit{A. baumannii} isolates (Table 1). The amplification products were sequenced for both strains.

IEF analysis. The \(\beta\)-lactamase extracts from cultures of \textit{O. urethralis} COH-1 and the \textit{E. coli} transformants were subjected to analytical isoelectric focusing (IEF) analysis on an ampholine polyacrylamide gel (pH 3.5 to 9.5; Ampholine Ceu; Ceu Biolabs, Saint-Quentin-en-Yvelines, France) as reported previously (4, 35). The agents and their sources have been described elsewhere (33). MICs were determined by an agar dilution kit, as described by the manufacturer (ECL; Amersham Pharmacia Biotech). The antimicrobial agents used in this study were obtained in the form of standard laboratory powders and were used immediately after their solubilization. The agents and their sources have been given accession numbers AY177427, AY178993, AY178995, and AY178996. The insertion sequence (IS) identified, IS\textit{Our1}, has been recorded and can be found on the Internet (http://www.is.biotoul.fr/).

TABLE 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>Sequence (5' to 3')*</th>
<th>Reference or source</th>
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<td>CARB-8A</td>
<td>\textit{bla}\textsubscript{CARB-8}</td>
<td>GGCATATTATGAGGCTTCTAG</td>
<td>This study</td>
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<td>CARB-8B</td>
<td>\textit{bla}\textsubscript{CARB-8}</td>
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<td>ampC-AB1</td>
<td>\textit{bla}\textsubscript{ampC}</td>
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<td>ampC-AB2</td>
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<tr>
<td>preABA-1</td>
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<td>This study</td>
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<td>preABA-2</td>
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<td>This study</td>
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<tr>
<td>ampC-AB3</td>
<td>\textit{bla}\textsubscript{ampC}</td>
<td>CTGTGTCTACTTATATCCCG</td>
<td>This study</td>
</tr>
<tr>
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<td>\textit{bla}\textsubscript{ampC}</td>
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<tr>
<td>A</td>
<td>16S RNA</td>
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<tr>
<td>B</td>
<td>16S RNA</td>
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<td>2</td>
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</table>

* H is A, T, or C; Y is C or T.

RESULTS

Cloning and nucleotide sequence analysis of \(\beta\)-lactamase-encoding genes. Cloning of \(\beta\)-lactam resistance markers of \textit{O. urethralis} COH-1 followed by their expression in \textit{E. coli} gave several recombinant plasmids including pOLI-1 and pOLI-2. These plasmids gave two \(\beta\)-lactam resistance phenotypes consistent with AmpC \(\beta\)-lactamase-type and penicillinase-type enzyme production, respectively. DNA sequence analysis of the 6-kb insert of pOLI-1 revealed an open reading frame (ORF) of 1,152 bp (Fig. 1) that shared 96% nucleotide identity with the \textit{ampC} gene of \textit{A. baumannii} RYC 52763/97 (5). The deduced amino acid sequence had five amino acid changes (98% amino acid identity) compared to the sequence of the cepha-

FIG. 1. Schematic map of part of the insert of recombinant plasmid pOLI-1 that encoded the \textit{bla}\textsubscript{ABA-1} gene. Open boxes, genes; arrows, translational orientations of the genes. Details of the nucleotide sequence of the putative promoter region of \textit{bla}\textsubscript{ABA-1} are given at the bottom. The boxed sequence corresponds to the left inverted repeat (IR\textsubscript{L}) of IS\textit{Our1}.
FIG. 2. Alignment of the amino acid sequences of the ABA-1 β-lactamase of *O. urethralis* COH-1 with the amino acid sequences of the cephalosporinases of *A. baumannii* clinical isolates ABAC1, ABAC2, and RYC 52763/67 (5). Dashes, identical amino acids. The serine β-lactamase motif S-V-S-K, the conserved triad K-T-G, and the class C typical motif Y-X-N are boxed in grey.
lossporinase of this *A. baumannii* strain (Fig. 2), but the changes did not likely modify its hydrolysis spectrum (31). The AmpC β-lactamase-type enzyme produced by strain COH-1 was designated ABA-1 (*for* *A. baumannii*).

Sequence analysis of the flanking DNA sequences of *bla*ABA-1 identified an IS element, designated ISOur1, in the region immediately upstream of the *bla*ABA-1 gene. The left inverted repeat of ISOur1 likely contained a −35 promoter region (TTGCAA) that may constitute a hybrid promoter together with a putative −10 promoter region (TATAAA), which was located downstream (Fig. 1). The −35 and −10 regions of this promoter were separated by a 17-bp sequence. ISOur1 had 83% and 73% nucleotide sequence identities with the sequences of IS1007 from *A. baumannii* LS56-7 (22) and IS26 from *Proteus vulgaris* (27), respectively, both of which belong to the IS6 family of IS elements. A 702-bp ORF within the ISOur1 sequence encoded a putative transposase that had 88% and 84% amino acid sequence identities with the transposase amino acid sequences of IS1007 and IS26, respectively.

According to the classification criteria of IS elements proposed by Mahillon and Chandler (25), ISOur1 belongs to the IS6 family. ISOur1 is 819 bp (750 to 900 bp) and has the amino acids that characterize the amino acid triad known as the DDE motif of the IS6 family.

Sequence analysis of PCR products obtained by PCR amplification with primers specific for the *A. baumannii* cephalosporinase gene and whole-cell DNAs of *A. baumannii* isolates ABAC1 and ABAC2 as templates yielded two novel β-lactamase-producing strains.* O. urethralis* COH-1 produced β-lactamases ABA-1 and CARB-8, whereas *E. coli* DH10B(pOLI-1) produced ABA-1 and *E. coli* DH10B(pOLI-2) produced CARB-8.

<table>
<thead>
<tr>
<th>β-Lactam</th>
<th>MIC (µg/ml)</th>
<th>O. urethralis COH-1</th>
<th>O. urethralis reference strains</th>
<th>E. coli DH10B(pOLI-1) (ABA-1)</th>
<th>E. coli DH10B(pOLI-2) (CARB-8)</th>
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<tr>
<td>Amoxicillin</td>
<td>512</td>
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<td>8</td>
<td>&gt;512</td>
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<td>8</td>
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<tr>
<td>Pipercillin</td>
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<td>16</td>
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<td>Cefoxitin</td>
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<tr>
<td>Cefepime</td>
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<td>Cefpirome</td>
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<td>Imipenem</td>
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<td>0.06</td>
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* O. urethralis COH-1 produced β-lactamases ABA-1 and CARB-8, whereas *E. coli* DH10B(pOLI-1) produced ABA-1 and *E. coli* DH10B(pOLI-2) produced CARB-8.

CLA, clavulanic acid at 2 µg/ml; TZB, tazobactam at 4 µg/ml.
sequences encoding cephalosporinases that had 98% amino acid identity with the amino acid sequence of *A. baumannii* RYC 52763/97 (5), indicating a high degree of identity of the sequences of the *A. baumannii* cephalexinases.

DNA sequence analysis of the 2.3-kb insert of the other recombinant plasmid, pOLI-2, identified an 897-bp ORF encoding a class A β-lactamase that had two amino acid substitutions compared to the amino acid sequence of carbenicillin-hydrolyzing β-lactamase CARB-5 from *A. baumannii* A85-145 (Fig. 3) (10). Amino acids of the consensus sequence of box VII of Ambler class A β-lactamases (20), at positions 234 to 236 (1), consisted of an RTG triad, as reported for the GN79 enzyme (RTG-1) from *Proteus mirabilis* GN79 (17) and the CARB-5 enzyme (RTG-2) from *A. baumannii* A85-145 (10, 30). The carbenicillin-hydrolyzing β-lactamase identified in O. urethralis COH-1 was designated CARB-8. Sequencing of the upstream and downstream regions (500 bp was analyzed in both cases) surrounding the *bla*<sub>CARBS</sub> gene did not identify any ORF.

**IEF analysis.** IEF analysis of a crude β-lactamase extract of isolate COH-1 gave two bands with pI values of 6.4 and 8.3 that comigrated with β-lactamases extracted from *E. coli* DH10B(pOLI-2) and *E. coli* DH10B(pOLI-1), respectively.

**Susceptibility testing.** *O. urethralis* COH-1 was resistant to amoxicillin, ticarcillin, cephalothin, and cefuroxime. Isolates with this resistance phenotype were partially antagonized by class A β-lactamase inhibitors (Table 2). The antibiotic resistance phenotype of *O. urethralis* COH-1 differed from those of three *O. urethralis* reference strains, which were fully susceptible to all β-lactams, consistent with a lack of β-lactamase expression (a negative result by the nitrocefin test; data not shown) (Table 2). Once the *bla*<sub>ABA-1</sub> gene was cloned and expressed in *E. coli*, it conferred resistance to amoxicillin, cephalothin, and cefuroxime and decreased susceptibility to ureidopenicillins and extended-spectrum cephalosporins, whereas it did not confer resistance to carboxypenicillin, which was consistent with data reported for the AmpC β-lactamase of *A. baumannii* (31). The MICs of β-lactams for *E. coli* DH10B(pOLI-2) encoding CARB-8 were consistent with those for strains with carbenicillin-hydrolyzing β-lactamases (24).

**Plasmid DNA extraction, conjugation assay, and transformation experiments.** Extraction of plasmid DNA from *O. urethralis* COH-1 failed. Similarly, transformation experiments and conjugation experiments also failed.

**Endonuclease restriction, electrophoresis, and hybridization experiments.** By using restriction enzymes *XbaI*, *SphI*, *ApaI*, and *SfiI*, whole-cell DNA of *O. urethralis* COH-1 yielded four distinct PFGE patterns (Fig. 4). Restriction digestion with the *DraI* enzyme failed. Five DNA fragments (1,100, 1,075, 1,050, 970, and 850 kb) were generated by using *CeuI* fragment (Fig. 5A). After transfer onto a nylon membrane, four of the five fragments (those of 1,100, 1,075, 970, and 850 kb) generated with *CeuI* fragment hybridized with a 16S rRNA-specific probe, whereas probes specific for *bla*<sub>ABA-1</sub> and *bla*<sub>CARBS</sub> hybridized with the 1,075- and 850-kb fragments, respectively (Fig. 5B, C, and D). Similarly, hybridization results with the PFGE gel containing restricted fragments indicated that the two β-lactamase genes were not located on the same restriction fragment (Fig. 4B and C). These results indicated the chromosomal and nonneighboring integration of the *bla*<sub>ABA-1</sub> and *bla*<sub>CARBS</sub> genes in *O. urethralis* COH-1.

**Distribution of *bla*<sub>CARBS</sub> and *bla*<sub>ABA-1</sub> in *O. urethralis*
strains. A search for the \textit{bla}_{\text{CARB-8}} and \textit{bla}_{\text{ABA-1}} genes in three \textit{O. urethralis} reference strains failed, which was consistent with the negative results of the nitrocefin test and the MICs of \beta-lactams for these strains (Table 2).

**DISCUSSION**

This work analyzed the \beta-lactamase content of an \textit{O. urethralis} isolate and is the first report of an acquired mechanism of antibiotic resistance in that species. The \beta-lactamase genes coding for a cephalosporinase and a carbenicillin-hydrolyzing \beta-lactamase were chromosomally encoded and were located on different chromosomal DNA fragments, suggesting that their acquisition by \textit{O. urethralis} may have corresponded to different genetic events.

The sequence of the \textit{bla}_{\text{ABA-1}} gene was almost identical to that of the \textit{ampC} gene of \textit{A. baumannii} (5). Additionally, the G+1 content (42%) of the 500-bp DNA sequence located downstream of the \textit{bla}_{\text{ABA-1}} gene corresponded to chromosomal DNA of \textit{A. baumannii} (6). Thus, an interspecies exchange of this \beta-lactam resistance marker had likely occurred, resulting in the chromosomal integration of the \textit{ampC} \beta-lactamase gene. This is one of the few examples of chromosomal integration of a \beta-lactamase gene known to be found naturally in another unrelated species. Chromosomal integration of a gene encoding an AmpC \beta-lactamase-type enzyme sharing a high degree of identity with the \textit{Citrobacter freundii} cephalosporinase has been reported in \textit{P. mirabilis} (7, 13).

Mobilization of cephalosporinase genes has been reported to be plasmid mediated (32). The events that have led to chromosomal integration of \textit{bla}_{\text{ABA-1}} in \textit{O. urethralis} COH-1 remain unknown. Theoretically, transformation (39), plasmid integration (as is known to occur in \textit{Pseudomonas aeruginosa} [28]), site-specific recombination, and transposition may have occurred.

A lack of a gene encoding an AmpR-like regulator upstream of \textit{bla}_{\text{ABA-1}} in \textit{O. urethralis} COH-1 may be related to the lack of an \textit{ampR}-like gene upstream of the \textit{bla}_{\text{ampC}} gene of \textit{A. baumannii}, as reported previously (5). The absence of AmpR was consistent with the noninducibility of \beta-lactam expression in \textit{O. urethralis} COH-1 (data not shown), as in \textit{A. baumannii}.

This work also provides the AmpC sequences of several \textit{A. baumannii} isolates and reports their high percentage of amino acid identity, as has also been reported, for example, for the cephalosporinases of \textit{P. aeruginosa} (11).

An element of the IS6 family, ISO\textit{tur}1, structurally related to IS\textit{ISO07} was identified in \textit{A. baumannii} LS56-7 (22) and was found immediately upstream of \textit{bla}_{\text{ABA-1}}. In the DNA sequence upstream of \textit{bla}_{\text{ABA-1}}, a putative hybrid promoter consisting of a −35 promoter located in the inverted repeat of ISO\textit{tur}1 and a −10 promoter sequence that may correspond to part of the original promoter sequence of a cephalosporinase gene of \textit{A. baumannii} was evidenced. Hybrid promoters containing a −35 promoter region located in the inverted repeat of the IS6 family of IS elements have been reported for \textit{bla}_{\text{TEM-6}} in \textit{Klebsiella pneumoniae} (15) and \textit{bla}_{\text{SHV-2a}} in \textit{P. aeruginosa} (28). Target site duplication that results from a transposition event was not found immediately downstream or upstream of ISO\textit{tur}1, suggesting that ISO\textit{tur}1 and \textit{bla}_{\text{ABA-1}} may have been cotransferred from \textit{A. baumannii}. This study provides another example of an association of an IS element with a class C \beta-lactamase gene (18, 19). Indeed, an ORF encoding a transposase related to that of IS\textit{ISOS} (of the IS91 family) was described upstream of the plasmid-mediated \textit{bla}_{\text{SHV-1}} cephalosporinase gene (18), and the insertion of IS2 has been reported as a source of a promoter for high-level expression of the chromosomally located \textit{ampC} gene in \textit{E. coli} (19).

The mechanism of acquisition of the \textit{bla}_{\text{CARB-8}} carbenicillin-hydrolyzing \beta-lactamase gene by \textit{O. urethralis} is also unknown. The CARB-8 \beta-lactamase is another representative of a subgroup of carbenicillin-hydrolyzing \beta-lactamases known as the “RTG subgroup,” which includes RTG-1 from \textit{P. mirabilis} GN79 (17), RTG-2 (CARB-5) from \textit{A. baumannii} AS5-145 (10), and now RTG-3 (CARB-8), for which the typical KTG motif of class A \beta-lactamases is replaced by an RTG motif. The CARB-8 \beta-lactamase likely has the same hydrolytic profile as CARB-5 (10). Additionally, identification of CARB-8 in \textit{O. urethralis} adds to the list of bacterial species from which carbenicillin-hydrolyzing \beta-lactamase genes have been isolated, being mostly gram-negative strict aerobes such as \textit{Vibrio cholerae} (9), \textit{Alcaligenes xylosoxidans} (12), \textit{A. baumannii} (10), \textit{P. aeruginosa} (11), and, more rarely, other members of the family \textit{Enterobacteriaceae} (35).

Finally, this work provides additional evidence of the transfer of an antibiotic resistance gene between phylogenetically unrelated bacterial species belonging to the same commensal flora (8). In the present case, the chromosomal integration of an \textit{ampC} gene of \textit{A. baumannii} may have stabilized the interspecies transfer in \textit{O. urethralis}.

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**REFERENCES**


