Isolation and Expression of a Novel Molecular Class D β -Lactamase, OXA-61, from *Campylobacter jejuni*

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A novel β -lactamase gene, *bla*_{OXA-61}, from *Campylobacter jejuni* GC015 was cloned and its nucleotide sequence determined. *bla*_{OXA-61} encodes a protein of 257 amino acids in which the active-site STFK tetrad and conserved class D β -lactamase motifs YGN and KTG were identified. A conserved sequence upstream of *bla*_{OXA-61} is required for expression in *Campylobacter*.

The majority of thermotolerant *Campylobacter* species, including *Campylobacter jejuni*, the leading cause of bacterial food-borne diarrheal disease throughout the world (6), show various levels of resistance to the antimicrobial agents penicillin G, ampicillin, amoxicillin, ticarcillin, and piperacillin (9). Although ampicillin is not considered useful for the treatment of *Campylobacter* infections (5), 83 to 92% of strains of *C. jejuni* produce β -lactamase (9, 16) and β -lactamase-positive strains have been reported to be significantly more resistant to amoxicillin, ampicillin, and ticarcillin than β -lactamase-negative strains (9).

Ambler molecular class D enzymes (oxacillinases) are active-site serine β -lactamases (11), are poorly inhibited by clavulanic acid (7), and generally have a preference for penam substrates including oxacillin (10). Oxacillinases may be chromosomally encoded (4); however, most oxacillinase genes form part of gene cassettes in class 1 integrons (14) and the frequent location of OXA genes on mobile genetic elements (plasmids or integrons) facilitates their spread (8). In *Campylobacter*, ampicillin resistance and the associated β -lactamase production are chromosomally encoded (17). This study describes the cloning and expression of a novel molecular class D β -lactamase structural gene, *bla*_{OXA-61}, from a human clinical strain of *C. jejuni*, GC015, isolated in 2001.

Bacterial strains and plasmids used in this study are listed in Table 1. Plasmids from both *Escherichia coli* and *C. jejuni* were prepared using the Nucleospin Plasmid kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. β -Lactam antibiotics used in this study were ampicillin, piperacillin, carbenicillin, potassium clavulanate, cephalothin, cefotaxime, meropenem, and imipenem. MICs of β -lactam antibiotics, with the exception of imipenem, were determined by agar dilution according to the National Committee for Clinical Laboratory Standards method (12). Imipenem MIC testing was performed using Etest strips. For *C. jejuni*, MIC testing was performed as previously described (1). Potassium clavulanate was used at a concentration of 2 μ g/ml.

To amplify the putative *C. jejuni* β -lactamase gene, primers

DBla-F and DBla-R (Table 2) were designed utilizing the sequence of the putative periplasmic class D β -lactamase gene reported in the *C. jejuni* ATCC 11168 genome sequence (GenBank accession no. NC_002163) and targeted the nucleotide sequences of the serine β -lactamase STFK (serine-X-X-lysine) active-site tetrad and the class D β -lactamase domain LKIS-X-X-EQ, respectively. A 310-bp PCR product, amplified from a crude lysate prepared from *C. jejuni* strain GC015, was ligated into pGU0202 (2), followed by CaCl_2 -mediated transformation into *E. coli* HB101 and sequencing using primers pDA2-F and pDA2-R (Table 2). Sequencing of products was performed using the ABI 377 sequencer (Applied Biosystems, Foster City, CA) after dye terminator cycle sequencing (ABI Prism BigDye terminator sequencing kit; Applied Biosystems). To determine the precise sequence of the putative β -lactamase gene, including its promoter region, inverse PCR was performed using ClaI -digested, self-ligated *C. jejuni* GC015 genomic DNA as a template. *Pfu* turbo proofreading DNA polymerase (Stratagene, La Jolla, CA) and inverse PCR primers Inv.D-F and Inv.D-R (Table 2), designed from the known sequence of the amplified segment of DNA, were used to amplify a 2.2-kb PCR product. Sequences were analyzed in silico to construct the entire GC015 putative β -lactamase DNA sequence.

An open reading frame of 774 bp was identified which encoded a putative 257-amino-acid protein, designated OXA-61. The serine β -lactamase active-site STFK tetrad (positions 58 to 61) and conserved motifs characteristic of molecular class D β -lactamase structural elements, YGN (positions 133 to 135) and KTG (positions 196 to 198), were identified (Fig. 1). The overall GC content of *bla*_{OXA-61} is 27.4%, which is similar to the overall GC content (30.6%) of the *C. jejuni* ATCC 11168 chromosome (15). Database searches carried out through the National Center for Biotechnology Information using the BLAST program (3) showed that the putative product of *bla*_{OXA-61} had only one amino acid substitution, Glu $\rightarrow$ Gly (position 202), compared to a putative class D β -lactamase from *C. jejuni* ATCC 11168 (GenBank accession no. NC_002163). A 122-bp region identified directly upstream of *bla*_{OXA-61} showed 99% identity with a region directly upstream of the *C. jejuni* ATCC 11168 putative class D β -lactamase gene. OXA-61 showed the highest identity to the oxacillinase FUS-1 (45%) from *Fusobacterium nucleatum* (GenBank acces-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Phenotype ^a	Reference or source
<i>E. coli</i> HB101	<i>thi-1 hsdS20</i> (r _B ⁻ m _B ⁻) <i>supE44 recA13 ara-14 leuB6 proA2 lacY1 rpsL20</i> (Str ^r) <i>xyL-5 mlI-1</i>	Sm ^r	Promega
<i>Campylobacter</i> strains			
<i>C. jejuni</i> GC015	Clinical strain	Amp ^r Carb ^r Pip ^r	This study
<i>C. jejuni</i> 428	β-Lactam-susceptible clinical strain		This study
Plasmids			
pGU0202	6.174-kb <i>E. coli</i> - <i>Campylobacter</i> shuttle vector; <i>aph</i> (3')-III	Km ^r	2
pGU0401 ^b	0.9-kb PCR product from <i>C. jejuni</i> GC015, cloned into pGU0202; <i>oxa-61</i> is in frame with <i>aph</i> (3')-III; includes 122-bp sequence upstream of <i>oxa-61</i>	Km ^r Amp ^r Carb ^r Pip ^r	This study
pGU0402 ^b	<i>oxa61</i> from pGU0401 cloned into pGU0202; <i>oxa-61</i> is out of frame with <i>aph</i> (3')-III; includes 122-bp sequence upstream of <i>oxa-61</i>	Km ^r Amp ^r Carb ^r Pip ^r	This study
pGU0403 ^b	<i>oxa61</i> from pGU0401 cloned into pGU0202; <i>oxa-61</i> , including putative -35 and -10 promoter regions, is in frame with <i>aph</i> (3')-III; 122-bp sequence upstream of <i>oxa-61</i> is truncated (70 bp)	Km ^r	This study

^a Sm, streptomycin; Amp, ampicillin; Km, kanamycin; Carb, carbenicillin; Pip, piperacillin.

^b Resistance phenotypes are those described for *C. jejuni* harboring recombinant plasmids pGU0401, pGU0402, and pGU0403.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide primer ^a	Application	Sequence ^b
DBla-F	PCR	5'-TTT <u>GGA TCC</u> CGC CAC TT-3'
DBla-R	PCR	5'-TTC <u>GGA TCC</u> GCT TAT TTT TAG T-3'
pDA2-F	Sequencing	5'-GAA GAA CAG TAT GTC GAG CTA-3'
pDA2-R	Sequencing	5'-TGT TGT GTG GAA TTG TGA GC-3'
Inv.D-F	Inverse PCR	5'-GAA GTG GCA AGA AGA ATT GG-3'
Inv.D-R	Inverse PCR	5'-CAT ATC TTG CGC CCA AGA AG-3'
D.2a.j-F	PCR	5'-AAT TTG GAT CCA TCG ATG GAT TG-3'
D.2a-R	PCR	5'-TAC GAA <u>GAG CTC</u> ACT AGC CAT C-3'

^a All oligonucleotide primers were purchased from Invitrogen Life Technologies.

^b Underlined nucleotides indicate the BamHI (GGATCC) and SacI (GAGCTC) restriction sites.

sion no. AAP69916) and also shares identity with OXA-10 (37%), OXA-23 (35%), and OXA-27 (34%) (GenBank accession no. AAB60534, AAS59523, and AAG35609, respectively).

Three plasmid constructs were generated utilizing the previously described shuttle vector pGU0202 (2) (Table 1) to determine whether *bla*_{OXA-61} was expressed using its own promoter sequences and whether the entire 122-bp sequence upstream of the β-lactamase gene was required for expression. Thus generated recombinant plasmids pGU0401, pGU0402, and pGU0403 were used to transform β-lactamase-negative *C. jejuni* strain 428, which does not harbor a homologue of *bla*_{OXA-61} (data not shown), and *E. coli* HB101. β-Lactam MICs for *E. coli* HB101 and *C. jejuni* 428 were then compared to those for *E. coli* and *C. jejuni* 428 carrying *bla*_{OXA-61} (Table 3).

Recombinant plasmids pGU0401 and pGU0402 conferred a ≥32-fold rise in the MICs of ampicillin, piperacillin, and carbenicillin in *C. jejuni* 428 cells, indicating a functional β-lactamase being expressed from its own promoter regions; resistance to cefotaxime and imipenem was not conferred. Consistent with the class D β-lactamases, clavulanic acid, at a concentration of 2 μg/ml, did not significantly inhibit hydrolysis of the penicillins by *bla*_{OXA-61}. The meropenem MIC increased fivefold; however, pGU0202 is a multicopy plasmid (>50 copies/cell) and weak carbapenemase activity has been reported previously from recombinant strains expressing cloned oxacillinase genes (14). Truncation of the sequence upstream of the *bla*_{OXA-61} putative -35 promoter region in recombinant plasmid pGU0403 resulted in reduction of β-lactam MICs to the same level as the β-lactam-susceptible, wild-type *C. jejuni* strain 428. RNA primer extension analysis is required to determine the transcription start point for *bla*_{OXA-61} in *C. jejuni*; the exact function of the 122-bp sequence upstream of *bla*_{OXA-61} remains to be determined.

In *E. coli*, the *bla*_{OXA-61} promoter region is only poorly recognized, resulting in weak transcription of the β-lactamase gene. A four- to eightfold rise in the carbenicillin MIC and a two- to fourfold rise in the ampicillin MIC were still conferred by *bla*_{OXA-61}, indicating a low level of transcription of *bla*_{OXA-61} from its promoter region in *E. coli*.

This study indicates that OXA-61 may contribute to resis-

TABLE 3. MICs of β -lactam antibiotics for *E. coli* HB101, *C. jejuni* GC015, *C. jejuni* 428, and OXA-61-producing transformants

Antibiotic + inhibitor ^b	MIC (μ g/ml) for the following strain (plasmid ^d):								
	<i>E. coli</i> HB101	<i>E. coli</i> HB101(pGU0401)	<i>E. coli</i> HB101(pGU0402)	<i>E. coli</i> HB101(pGU0403)	<i>C. jejuni</i> GC015	<i>C. jejuni</i> 428	<i>C. jejuni</i> 428(pGU0401)	<i>C. jejuni</i> 428(pGU0402)	<i>C. jejuni</i> 428(pGU0403)
Ampicillin + CLA	4	16	8	8	64	2	128	128	2
	4	16	— ^c	—	32	<0.5	64	—	—
Piperacillin + CLA	1	4	1	1	>256	16	>256	>256	32
	1	4	—	—	256	8	256	—	—
Carbenicillin + CLA	8	64	32	32	128	8	256	128	8
	4	32	—	—	64	4	64	—	—
Cephalothin	4	16	—	—	—	—	—	—	—
Cefotaxime	<0.5	<0.5	—	—	4	4	4	—	—
Imipenem	0.125	0.125	0.125	0.125	0.064	0.064	0.064	0.064	0.064
Meropenem	0.032	0.032	0.032	0.032	0.125	0.064	2	2	0.064

^a pGU0401 harbors *bla*_{OXA-61} in frame with proximal *aph*(3')-III and includes 122-bp sequence upstream of *bla*_{OXA-61}. pGU0402 harbors *bla*_{OXA-61} out of frame with proximal *aph*(3')-III and includes 122-bp sequence upstream of *bla*_{OXA-61}. pGU0403 harbors *bla*_{OXA-61} in frame with proximal *aph*(3')-III; the 122-bp sequence upstream of *bla*_{OXA-61} has been truncated to remove 70 bp upstream of the putative -35 promoter region.

^b CLA, clavulanic acid (2 μ g/ml).

^c —, Not determined.

tance to β -lactams in *C. jejuni*. Future biochemical analyses will determine its functional classification according to the scheme of Bush et al. (7).

Nucleotide sequence accession number. The nucleotide sequence of *bla*_{OXA-61} has been deposited in GenBank under accession number AY587956.

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