Analysis of a carbapenem-hydrolyzing class A β -lactamase from *Enterobacter cloacae* and of its LysR-type regulatory protein

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Communicated by Werner Arber, April 13, 1994 (received for review January 3, 1994)

ABSTRACT Carbapenems such as imipenem are extended-spectrum β -lactam antibiotics, which are not hydrolyzed by the β -lactamases commonly found in Enterobacteriaceae. Here we report a gene encoding a carbapenemase, which was cloned from the chromosome of a clinical isolate of *Enterobacter cloacae*, strain NOR-1, into pACYC184 plasmid in *Escherichia coli*. Unlike all the sequenced carbapenemases, which are class B metallo- β -lactamases, the mature protein (Nmca) is a class A serine β -lactamase. Nmca shares the highest amino acid identity (50%) with the extended-spectrum class A β -lactamase MEN-1 from *E. coli*. In the opposite orientation from the *nmca* promoter, an overlapping and divergent promoter was detected, along with an open reading frame, which encoded a 33.5-kDa protein (Nmcr). The Nmcr amino acid sequence displays homology with LysR-type transcriptional regulatory proteins, including the conserved residues near its N terminus within a helix–turn–helix motif. Deletion of *nmcr* resulted in decreased carbapenem resistance and a loss of β -lactamase inducibility, demonstrating a positive role of Nmcr in Nmca expression.

β -Lactamase production is the most common mechanism of resistance to β -lactam antibiotics in Gram-negative bacteria. Although numerous β -lactamases have been described as efficiently hydrolyzing almost all commercially available β -lactam drugs, including extended-spectrum cephalosporins, carbapenems such as imipenem are not significantly hydrolyzed by enterobacterial β -lactamases. Carbapenems are active against both Gram-positive and Gram-negative organisms, including aerobic and anaerobic fermenters and nonfermenters. Such an extended spectrum of activity is not found in penicillin, cephalosporin, or monobactam antibiotics. These properties make carbapenems highly effective for treating infections due to Gram-negative nosocomial pathogens.

Based on protein sequence analysis, four β -lactamase classes have been identified by Ambler (1). They comprise the following: class A, widespread chromosomal or plasmid-mediated penicillinases; class B, rare metalloenzymes (mainly chromosomally located); class C, mainly chromosomal cephalosporinases; class D, plasmid-mediated oxacillinases. Carbapenemases have been studied at the genetic level in only a limited number of species of minor clinical importance such as *Bacteroides fragilis* (two strains; ref. 2), *Bacillus cereus* (two strains; ref. 3), and *Aeromonas hydrophila* (one strain; ref. 4). The activity of these class B metalloenzymes is zinc dependent and inhibited by EDTA.

Genes encoding resistance to most other β -lactams have been transferred within bacterial populations and are now widely distributed in enterobacterial species. We have previously described a carbapenem-hydrolyzing β -lactamase isolated from an enterobacterial species, *Enterobacter clo-*

acae NOR-1 (5). Hybridization studies indicated that this gene was unlike common Enterobacteriaceae β -lactamase and carbapenemase genes. Should this carbapenem resistance spread similarly, it would seriously compromise the effective use of carbapenems in clinical therapy. Thus, understanding more about this unusual β -lactamase and the mechanisms that regulate its expression may be of critical importance for the development of resistant β -lactams.[§]

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. cloacae* NOR-1, which produces the Nmca (not metalloenzyme carbapenemase A) β -lactamase, was isolated in Paris, France (5). A recombinant plasmid, pPTN1, which contains a 5.3-kb *Sau3A* fragment from the chromosome of *E. cloacae* NOR-1 cloned into the *Bam*HI site of pACYC184, has been described (5). pPTN3 was constructed by inserting a 2.2-kb *Nae* I fragment from pPTN1 into the *Sma* I site of pK19 (6) (see Fig. 5). The role of the surrounding DNA sequences in β -lactamase biosynthesis was studied by deleting parts of the inserted *Nae* I fragment in pPTN3. Subclonings were performed by inserting either the *Hinc*II/*Nae* I fragment (pPTN4), the *Nco* I/*Nae* I fragment (pPTN5), or the *Nde* I/*Nae* I fragment (pPTN6) from pPTN3 into pK19 by standard molecular techniques.

Minimum Inhibitory Concentrations (MICs) and β -Lactamase Activity. Basal and ampicillin (100 μ g/ml)-induced β -lactamase activities as well as MICs were determined as described (5).

DNA Sequencing and Protein Analysis. DNA sequencing was completed on both strands by the dideoxynucleotide chain-termination method (7). The nucleotide sequence and the deduced protein sequences were analyzed with Genetics Computer Group (GCG) software (UW-Madison, Biotechnology Center, Madison, WI). Known DNA and protein sequences used for comparison of β -lactamases and LysR-type proteins were from EMBL and Swiss-Prot data bases. Multiple alignment of peptide sequences was carried out with the GCG program PILEUP. A phylogenetic tree was constructed from multiple β -lactamase alignments by a parsimony method using the phylogeny package PAUP (8).

Transcription Start of *nmca* and *nmcr*. mRNAs were extracted and analyzed as described (9) from *Escherichia coli* JM109 (pPTN1) and *E. cloacae* NOR-1.

N-Terminal Protein Sequencing. The size of the Nmca leader peptide was determined with *E. coli* JM109 and *E. cloacae* NOR-1, both transformed by pPTN3 and grown with ampicillin (100 μ g/ml). Crude extracts of both ampicillin-induced bacteria contained a major well-separated band of the same size on SDS/polyacrylamide gels, which had a β -lactam-hydrolyzing activity as revealed by overlaying the

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Abbreviations: MIC, minimum inhibitory concentration; ORF, open reading frame.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. Z21956).

peptide (Fig. 1). Thus, NmcA mature protein had a mass of 29.10 kDa (265 amino acids).

In the opposite orientation from the *nmcA* ORF, a second ORF encoded a 33.44-kDa protein (295 amino acids for the presumed mature protein) named NmcR (not metalloenzyme carbapenemase regulator), assuming that the ATG at nucleotide positions 1077–1079 is the start codon (Fig. 1). mRNA primer-extension analysis revealed a presumed transcriptional start site at position 1107 in Fig. 1 for *nmcR* in *E. cloacae* NOR-1 and in *E. coli* JM109 (pPTN1). A typical Enterobacteriaceae promoter sequence was found upstream of this initiation codon containing a –35 region (TTGTTA) and a –10 region (TATAAT) separated by 17 bp. This promoter overlapped the *nmcA* promoter. A palindromic sequence (a1 and a2 in Fig. 1) downstream of this ORF encodes a potential stem-loop structure, which presumably functions as a Rho-independent transcription terminator ($\Delta G = -12$ kcal/mol at 25°C).

The overall G+C content of *nmcA* (39%) and *nmcR* (40%), as well as the corresponding codon usage, was typical of Enterobacteriaceae genes (12). The organization of the promoters of these two genes was similar to that of other Enterobacteriaceae β -lactamase gene regulatory systems such as the *ampR-ampC* of *Citrobacter freundii*, *E. cloacae*, and *Yersinia enterocolitica* (Fig. 2) (13).

NmCA Homology with Other β -Lactamases. The nucleotide sequence of the NmCA structural gene had <40% identity with all other β -lactamase genes. Comparison of deduced amino acid sequences revealed that NmCA had very weak identity with class B metalloenzyme β -lactamases (18% with

The *nmcR-nmcA* -35 and -10 promoter regions (Fig. 2; ref. 13) are similar to *ampR-ampC* regions from various Enterobacteriaceae. As recently described for the putative AmpR binding sequences of the *C. freundii*, *Y. enterocolitica*, and *E. cloacae ampR-ampC* systems, two regions were found for *nmcR-nmcA* (Fig. 2). Region 1 corresponded to the 5' half of the putative protected region relative to the *nmcA* promoter and contained a partial palindromic sequence, separated by a 3- to 5-bp A+T-rich region. Region 2 corresponded to the 3' half of the putative protected region and also contained palindromic base pairs but without a spacer region. Each half of the palindromic sequence at region 1 showed partial homology with the corresponding half of region 2. In region 1 but not in region 2 there was a LysR motif consisting of T-11 nucleotides-A around a twofold axis of symmetry (29). AmpR of *C. freundii* binds only to region 1. The questions remaining to be solved concerning NmcR include whether NmcR regulates its own synthesis as de-

such as *cfiA* from *B. fragilis*, *cphA* from *A. hydrophila*, and from the *B. cereus* type II β -lactamase gene, showed no similarity even in sequences thought to form the catalytic site (data not shown). Conversely, NmcA shared identical amino acids with class A β -lactamases in its catalytic site that

FIG. 5. Restriction endonuclease map of recombinant plasmids that contained deletions within the *nmcR* sequence. Corresponding imipenem MICs and ampicillin (100 μ g/ml) β -lactamase inducibility, as measured in *E. coli* JM109 harboring these plasmids, are also shown (5). Activities were measured after 5 hr of growth in 500 ml of LB broth at 37°C (5). Deletions were performed (broken line) in the *Nae* I fragment, which contains the entire *nmcR*–*nmcA* genes as shown, and subcloned in pK19.

interact with β -lactam compounds—i.e., in boxes I–VII and the SDN motif. Although all seven boxes are highly conserved in class A β -lactamases, boxes III, V, and VII, plus the SDN motif, are especially critical for the activity of the enzyme. Inspection of the NmcA sequence suggested that its hydrolytic properties toward imipenem may result from the presence of specific residues. In box III, the presence of a histidine residue (position ABL 105) has not yet been described in any other class A β -lactamase, and this sequence may be involved, in part, in the carbapenem-hydrolyzing property of NmcA. Moreover, close to box VII (positions ABL 234–236), the presence of a serine residue followed, at position ABL 239, by a cysteine could also be involved in the unusual catalytic properties of NmcA. Dendrogram analysis (data not shown) revealed that NmcA may be part of the class A β -lactamase subgroup (YENT, CITDI, KOXY, and MEN-1) that comprises enterobacterial chromosomally encoded β -lactamases (19).

As with the class A β -lactamase isolated from *S. cacaoi* (26), a regulator, NmcR, in *E. cloacae* NOR-1 acted as a positive regulator for carbapenemase biosynthesis, even in the absence of any inducer. This property is very rare among LysR regulatory systems, where an inducer is usually required for positive regulation. In the AmpR–AmpC system, AmpR differs from NmcR in that it acts as a negative regulator of the cephalosporinase expression in the absence of inducer and as a positive regulator in the presence of inducer. It is unlikely that β -lactams themselves act as inducers for the AmpR–AmpC system or NmcR–NmcA, since they probably do not cross the cell membrane (13). Peptidoglycan degradation products may act as inducers in both cases. In this regard, the mature NmcR protein did not contain hydrophobic regions, which are usually found in transmembrane proteins. Deletion of the C terminus of NmcR led to a loss of its positive regulator properties, indicating its role in protein structure maintenance or as an inducer sensor. Finally, this work identified a regulatory system of a class A β -lactamase in Enterobacteriaceae. In a recent review, Livermore (30) suggests that carbapenemases may be the next generation of β -lactamases; NmcA is the

only such enzyme to be sequenced from an opportunistic nosocomial pathogen belonging to the Enterobacteriaceae.

We are grateful to W. Arber and C. Nauciel, in whose laboratory part of this work was performed. This study was supported by the Fondation pour la Recherche Médicale, France. We thank V. Pajadon for providing us a grant-in-aid from Merck-Sharp and Dohme-Chibret, France.

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