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Chromobacterium spp. harbour Ambler class A β-lactamases showing high identity with KPC

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Objectives: The origin of KPC is unknown. The aim of this study was to detect progenitors of KPC *in silico* and to functionally verify their β-lactam hydrolysis activity.

Methods: The sequence of KPC-2 was used to mine the NCBI protein sequence database. The best non-KPC hits were analysed by amino acid (aa) alignment and phylogenetic tree construction. Genes encoding KPC-2 homologues were expressed in *Escherichia coli*. The carbapenemase activities of the recombinant strains were characterized by the CarbaNP test and UV spectrophotometry and MICs of selected β-lactams were determined.

Results: Genes encoding the closest KPC-2 homologues were identified on the chromosome of *Chromobacterium piscinae* strain ND17 (CRP-1, 76% aa identity), *Chromobacterium* sp. C-61 (CRS-1, 70% aa identity) and *Chromobacterium haemolyticum* DSM19808 (CRH-1, 69% aa identity). All three *Chromobacterium* β-lactamases were phylogenetically more related to KPC than to other Ambler class A β-lactamases. The 27 bp region preceding the start codon of bla_{CRP-1} displayed high nucleotide identity to the corresponding region upstream from bla_{KPC} (74%). Heterologous expression of bla_{CRP-1} and to a lesser extent of bla_{CRH-1} in *E. coli* significantly increased the MICs of meropenem and most cephalosporins. The CarbaNP test was positive for both recombinant strains, but spectrophotometric analysis confirmed higher carbapenemase activity for CRP-1-producing clones.

Conclusions: The recovery of three class A β-lactamases with up to 76% aa identity to KPC from distinct *Chromobacterium* species is highly indicative of the role played by this genus in the evolution of KPC.

Introduction

Class A carbapenemases are serine-based β-lactamases that can hydrolyse almost all clinically used β-lactams including carbapenems, thus representing a therapeutic challenge when acquired by pathogenic bacteria.[1] Class A carbapenemases have been reported in Gram-negative bacteria from a wide variety of environments ranging from rivers to hospitals.[2,3] The corresponding genes may be located on the chromosome, but are mainly plasmid-borne when acquired.[1,3]

KPC is the most frequently detected plasmid-borne class A carbapenemase in Enterobacteriaceae.[1,3] Twenty-three KPC variants have been described since the discovery of the first KPC enzyme in the USA in 2001[4] with KPC-2 being the most prevalent variant worldwide.[5] The bla_{KPC} gene is usually located on transposon Tn*4401*, a Tn*3*-like transposon located on conjugative plasmids of different size.[6,7] Five Tn*4401* isoforms (Tn*4401a* to Tn*4401e*) have been identified mainly based on DNA polymorphism in the region upstream of bla_{KPC}.[6,8] The origin of KPC is unknown, but it has been suggested that Tn*4401* might be responsible for the mobilization of bla_{KPC} from the chromosome of an unknown bacterial host to plasmids of various size and nature.[6]

The aim of this study was to explore the NCBI protein sequence database for the presence of possible KPC progenitors. The best candidates were expressed and characterized functionally in *Escherichia coli*.

Materials and methods

NCBI protein sequence database mining

The KPC-2 sequence was entered as a query into the blastp algorithm at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using all default parameters. In order to detect the closest KPC homologues, all hits were preliminarily analysed by the NCBI inbuilt alignment and phylogenetic tree analysis tools.[9] Sequences of the closest KPC-2 homologues were aligned with previously described class A carbapenemases[3] including KPC-2, NMC-A, SFC-1, IMI-1 and SME-1 in order to identify possible conserved residues critical for carbapenemase activity. Alignments were performed by the ClustalW

program inbuilt into MEGA6 (http://www.megasoftware.net/). A phylogenetic tree was constructed by the neighbour-joining method and evolutionary analysis was conducted in MEGA6 (http://www.megasoftware.net/). Aligned sequences were visualized by ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/).

Strains, media and antibiotics

Chromobacterium haemolyticum DSM19808 strain was obtained from sputum and belongs to the DMSZ strain collection (Braunschweig, Germany). *Chromobacterium piscinae* ND17 originated from water and was donated by Chan Kok Gan, University of Malaya, Malaysia. *Chromobacterium* sp. C-61 originated from *Solanum melongena* rhizosphere and was donated by Young Cheol Kim, Chonnam National University, Republic of Korea. All media and antibiotics were purchased from Difco™ (Le Pont-de-Claix, France) and Sigma–Aldrich (Steinheim, Germany), respectively.

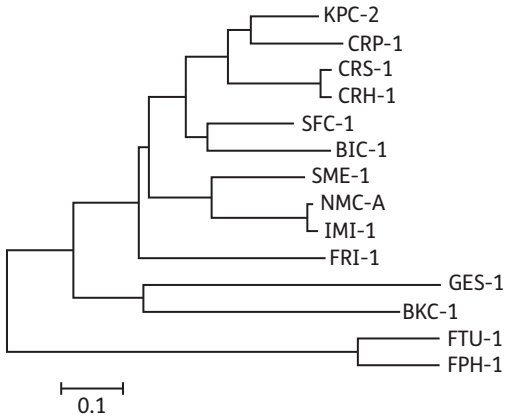

Figure 1. Phylogenetic tree showing the evolutionary relationship between the three *Chromobacterium* β-lactamases described in this study and previously described Ambler class A carbapenemases. The tree is drawn to scale and the evolutionary distances are in units of aa substitutions per site. GenBank accession numbers: CRP-1, WP_043629745.1; CRH-1, WP_043592266.1; CRS-1, WP_039755574.1; KPC-2, AY034847; SFC-1, AY354402; BIC-1, GQ260093; NMC-A, Z21956; IMI-1, U50278; SME-1, U60295; GES-1, AAL82589; FTU-1, YP_513599.1; FPH-1, ZP_05249935.1; FRI-1, KT192551; and BKC-1, KP689347.

Functional characterization of KPC homologues in E. coli

Putative class A carbapenemase-encoding genes from *C. piscinae* ND17 and *C. haemolyticum* DSM19808 were cloned into plasmid pZE21MCS, transformed into One Shot® TOP10 Electrocomp™ *E. coli* (Invitrogen, Carlsbad, CA, USA)[10] and subjected to confirmatory sequencing (Macrogen, Republic of Korea). Recombinant clones were selected on LB agar containing 30 mg/L amoxicillin and 50 mg/L kanamycin (resistance marker for pZE21MCS). Carbapenemase activity was determined by the CarbaNP test[11] and quantified by measuring the imipenem hydrolysis rate by UV spectrophotometry.[12] MICs of different β-lactams were measured for the *E. coli* recombinant strains by broth microdilution using Sensititre ESBL plates (Trek Diagnostic Systems, USA). If the precise MIC of an antimicrobial was not within the range included in the Sensititre ESBL plate, broth microdilution was performed manually according to CLSI guidelines. MICs for the *Chromobacterium* strains were determined by Etest (bioMérieux, Marcy-l'Étoile, France) using standard culture conditions.

Results

The hits obtained by blasting KPC-2 comprised in sequential order all known KPC enzymes and putative class A β-lactamases in members of the genus *Chromobacterium*. Phylogenetic analysis indicated that the putative class A β-lactamases from *C. piscinae* (GenBank accession number WP_043629745.1), *Chromobacterium* sp. C-61 (WP_039755574.1) and *C. haemolyticum* (WP_043592266.1) were more closely related to KPC-2 than to other class A carbapenemases (Figure 1). The β-lactamase-encoding gene from *C. piscinae* (bla_{CRP-1}) had more similar GC content (64%) and higher nucleotide identity (76%) with bla_{KPC-2} (GC content 61%) than those from *C. haemolyticum* (bla_{CRH-1}; 70% nucleotide identity and 65% GC content) and *Chromobacterium* sp. C-61 (bla_{CRS-1}; 68% nucleotide identity and 66% GC content). The amino acid (aa) identities of CRP-1, CRH-1 and CRS-1 with previously described class A carbapenemases are shown in Table S1 (available as Supplementary data at *JAC* Online). Sequence alignment displayed minor heterogeneities at the N- and C-termini, but residues deemed essential for carbapenemase activity were conserved in all three enzymes except for the H105W substitution, which is present in 15 out of the 19 KPC variants for which sequences are publicly available (Figure S1).

Table 1. Carbapenemase activity and MICs of β-lactams for WT *E. coli* TOP10 (WT) and recombinant *E. coli* TOP10 clones producing class A β-lactamases from *C. piscinae* ND17 (pCRP-1) or *C. haemolyticum* DSM19808 (pCRH-1)

		MIC (mg/L) of β-lactams													
Strain	Carbapenemase activity[a]	AMP (>8)	FOX (NA)	CFZ (NA)	CEF (NA)	CTX (>2)	CAZ (>4)	CPD (>1)	CRO (>2)	FEP (>4)	CTX/CLA (NA)	CAZ/CLA (NA)	TZP (NA)	IPM (>8)	MEM (>8)
WT	<1	≤8	8	0.5	≤8	0.01	0.06	0.5	0.01	0.125	≤0.12	≤0.12	≤4	0.03	0.125
pCRP-1	186	>16	32	>16	>16	1	4	4	4	0.25	0.5	1	64	0.25	4
pCRH-1	27	>16	8	>16	>16	0.25	0.5	1	0.5	0.125	≤0.12	≤0.12	≤4	0.06	0.5

AMP, ampicillin; FOX, cefoxitin; CFZ, cefazolin; CEF, cefalotin; CTX, cefotaxime; CAZ, ceftazidime; CPD, cefpodoxime; CRO, ceftriaxone; FEP, cefepime; CTX/CLA, cefotaxime/clavulanic acid; CAZ/CLA, ceftazidime/clavulanic acid; TZP, piperacillin/tazobactam; IPM, imipenem; MEM, meropenem; NA, not available.

When available, the EUCAST clinical breakpoint for Enterobacteriaceae is indicated in parentheses (www.eucast.org).

[a]Carbapenemase activity was defined as 1 μmol of imipenem hydrolysed/min/mg of protein.

Alignment of the sequences surrounding the three bla_{KPC} homologues identified distinct genetic contexts and displayed no nucleotide identity to the corresponding regions in the Tn*4401* isoforms with the exception of the 148 bp non-coding region upstream of bla_{CRP-1}, which displayed 74% nucleotide identity over 27 bp and 100% nucleotide identity over 11 bp (Figure S2).

Meropenem MICs for *C. piscinae*, *C. haemolyticum* and *Chromobacterium* sp. C-61 were 0.5, 0.75 and 0.5 mg/L, respectively. Carbapenemase activity was confirmed in *C. haemolyticum* and *Chromobacterium* sp. C-61 (13 and 20 μmol of imipenem hydrolysed/min/mg of protein, respectively). Spectrophotometric analysis detected notable carbapenemase activity for CRP-1-producing clones as compared with CRH-1-producing clones (Table 1). Heterologous expression of bla_{CRP-1} in *E. coli* significantly increased the MICs of carbapenems and most cephalosporins, often above the EUCAST clinical breakpoints (www.eucast.org), whereas lower increases in the MICs were observed for the CRH-1-producing clones (Table 1). Carbapenemase activity was detected by the CarbaNP test in both recombinant clones.

Discussion

We identified three distinct *Chromobacterium* species harbouring genes encoding functional class A β-lactamases with high similarity to KPC with regard to aa residue composition, sequence length, aa identity and phylogenetic position. Based on aa identity (≤76%), it is unlikely that KPC may have originated directly from any of the three species described in this study. However, our results indicate that the genus *Chromobacterium* may have played a role in the evolution of KPC.

C. piscinae and *C. haemolyticum* are commonly isolated from aquatic environments.[13,14] The latter species has been associated with bacteraemia in healthy individuals with a history of freshwater exposure and is known for being high-level resistant to ampicillin and amoxicillin/clavulanic acid.[13] Various Gram-negative bacteria living in aquatic environments have been shown to produce other class A carbapenemases such as BIC-1, SFC-1 and IMI-2,[2,15,16] suggesting that aquatic environments constitute a reservoir of novel carbapenem resistance genes for clinically relevant bacteria.

No mobile genetic elements were detected in the regions flanking bla_{CRP-1}, bla_{CRH-1} and bla_{CRS-1}, suggesting that these genes are normally present in the chromosome of *Chromobacterium* species. This hypothesis is further supported by their GC content (64%–66%), which is similar to that of the whole *Chromobacterium* chromosome (63%–66%).

Notably, all three *Chromobacterium* strains were susceptible to meropenem (MIC = 0.5–0.75 mg/L). Similar observations have previously been reported for other carbapenemase-encoding genes of environmental origin such as bla_{OXA-23} in *Acinetobacter radioresistens*[17] and $bla_{OXA-48-like}$ in *Shewanella* species.[18,19] Upon mobilization and subsequent dissemination to other species (involving insertion of sequences and high-copy plasmids), these resistance genes may become functional in heterologous bacterial hosts by exogenous promoters and multiple gene copy numbers. The reason why some environmental bacteria harbour intrinsic carbapenemase-encoding genes remains unknown, especially when such genes seem to be not (or very poorly) expressed under laboratory conditions.

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Transparency declarations

None to declare.

Supplementary data

Table S1, Figure S1 and Figure S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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