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Heterogeneous hydrolytic features for OXA-48-like β-lactamases

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Objectives: Carbapenem-hydrolysing class D β-lactamases of the OXA-48 type are increasingly reported from Enterobacteriaceae. β-Lactamase OXA-48 hydrolyses penicillins very efficiently, but carbapenems only weakly and spares broad-spectrum cephalosporins. Recently, diverse OXA-48-like β-lactamases have been identified worldwide (OXA-162, OXA-181, OXA-163, OXA-204 and OXA-232). They differ by few amino acid substitutions or by amino acid deletions.

Methods: $bla_{\text{OXA-48}}$, $bla_{\text{OXA-162}}$, $bla_{\text{OXA-163}}$, $bla_{\text{OXA-181}}$, $bla_{\text{OXA-204}}$ and $bla_{\text{OXA-232}}$ were cloned into the same expression vector and expressed in the same *Escherichia coli* background. Kinetic studies were performed with enzymes purified by ion-exchange chromatography. Determination of hydrolytic activities was performed by UV spectrophotometry. MICs were determined for all recombinant strains, using as background either the WT *E. coli* TOP10 strain or a porin-deficient *E. coli* strain.

Results: Kinetic studies showed that OXA-162 and OXA-204 shared the same hydrolytic properties as OXA-48. On the other hand, OXA-181 possessed a higher ability to hydrolyse carbapenems, while OXA-232 hydrolysed those substrates less efficiently. In contrast to the other OXA-48-like β-lactamases, OXA-163 hydrolysed broad-spectrum cephalosporins very efficiently, but did not possess significant carbapenemase activity. Although several of these OXA-48-like enzymes possess low activity against carbapenems, MICs of carbapenems were significantly elevated when determined for strains possessing permeability defects.

Conclusions: A detailed comparative analysis of the kinetic properties of the OXA-48-like β-lactamases is provided here. It clarifies the respective features of each OXA-48-like variant and their respective impacts in terms of carbapenem resistance.

Keywords: carbapenemases, class D β-lactamases, enzymatic activity

Introduction

The emerging mechanism of resistance to carbapenems in Enterobacteriaceae is related to the horizontal transfer of plasmid-mediated carbapenemase genes.[1,2] Carbapenemases belong to the Ambler class A, B or D β-lactamases.[3] OXA-48 and its derivatives are class D β-lactamases and have disseminated widely, but only in Enterobacteriaceae.[4,5] They are widespread in particular in Europe, Africa and the Indian subcontinent.[1] β-Lactamase OXA-48 hydrolyses penicillins at a high level and carbapenems at a low level, but spares expanded-spectrum cephalosporins such as ceftazidime.[5] Due to these properties, OXA-48 producers may be either susceptible or resistant to broad-spectrum cephalosporins (additional production of an ESBL observed in 80% of strains[6,7]) and to carbapenems. Indeed, additional mechanisms such as permeability defects, efflux overproduction and high-level production of expanded-spectrum β-lactamases (AmpC and ESBLs) have been shown to confer reduced susceptibility to carbapenems when combined.[5]

Since the first identification of OXA-48,[8] different variants have been reported, differing by few amino acid substitutions or deletions.[5] Whereas some OXA-48-related enzymes such as OXA-48, OXA-162, OXA-181, OXA-204 and OXA-232 have been reported as conferring a very similar resistance pattern, OXA-163 (with a single amino acid substitution together with a four amino acid deletion compared with OXA-48) compromises the efficacy of broad-spectrum cephalosporins and hydrolyses carbapenems only marginally.[9] From a biochemical point of view, these OXA-48-like β-lactamases have not been purified and analysed under the same conditions. This may create misleading interpretations when comparing the respective kinetic data, since values are known to vary under different experimental conditions (e.g. buffer content and concentration for hydrolysis assays, reaction temperature and type of UV spectrophotometer). Similarly, since expression of the different bla_{OXA-48}-like genes has been evaluated using different cloning vectors and different *Escherichia coli* host strains, MIC interpretations might again be misleading when comparing the susceptibility of the different recombinant strains. Therefore, and in order to gain further insights into the relative hydrolytic properties of these different variants, we designed a study aiming to clone and express in the same genetic background a series of bla_{OXA-48}-like genes. Here, we evaluated the susceptibility of the corresponding recombinant clones to β-lactams, including temocillin, which is reported to be a good substrate of OXA-48 and therefore used as a marker for the detection of OXA-48-like producers.[10] Additionally, the relative catalytic activities of purified enzymes and the performance of the Carba NP test were tested for these OXA-48-like β-lactamases.

Materials and methods

Bacterial strains

A series of clinical *Klebsiella pneumoniae* isolates harbouring bla_{OXA-48}-like genes were used as templates. These isolates respectively harboured genes encoding OXA-162 (France, 2013),[7] OXA-163 (Argentina, 2011),[9] OXA-181 (France 2013),[11] OXA-204 (France, 2013)[12] and OXA-232 (India, 2012).[13] These variants differed from OXA-48 by a single amino acid substitution or by amino acid deletions (Figure 1). *E. coli* TOP10 was used as a recipient strain for cloning and expressing the different bla_{OXA-48}-like genes as described previously.[12] In addition, *E. coli* HB4 lacking the major porins OmpF and OmpC was also used as a recipient strain to evaluate the relative impact of the expression of these genes in an *E. coli* reference strain with low-level outer membrane permeability.[14]

Cloning and expression of the bla_{OXA-48}-like genes

The entire coding sequences of the β-lactamase genes ($bla_{OXA-162}$, $bla_{OXA-163}$, $bla_{OXA-181}$, $bla_{OXA-204}$ and $bla_{OXA-232}$) were obtained by PCR amplification using primers OXA-48A (5′-TTGGTGGCATCGATTATCGG-3′) and OXA-48B (5′-GAGCACTTCTTTTGTGATGGC-3′) and were then inserted into plasmid pCR®-Blunt II-TOPO® (Invitrogen, Illkirch, France) for the phenotypic studies and pET9a (Novagen, VWR International, Fontenay-sous-Bois, France) following the manufacturer's recommendations. Recombinant plasmids obtained with pCR®-Blunt II-TOPO® were transformed into either *E. coli* strain TOP10 or *E. coli* strain HB4. The orientation of the respective inserts was checked by sequencing to ensure all genes were under the control of the P_{lac} promoter. Recombinant plasmids constructed in plasmid pET9a (Stratagene, Amsterdam, the Netherlands) used as expression vector and then were transformed into *E. coli* strain BL21(DE3) (Novagen) following the manufacturer's recommendations.

β-Lactamase purification

An overnight culture of *E. coli* strain BL21 harbouring pET9a-derived recombinant plasmids was used to inoculate 2 L of LB medium broth containing 50 mg/L kanamycin. Bacteria were cultured at 37°C until reaching

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                      10         20        30       40       50       60        70         80       90
                       *          *         *        *        *        *         *          *        *
OXA-48      MRVLALSAVFLVASIIGMPAVAKEWQENKSWNAHFTEHKSQGVVVLWNENKQQGFTNNLKRANQAFLPASTFKIPNSLIALDLGVVKDEHQ
OXA-162     -------------------------------------------------------------------------------------------
OXA-163     -------------------------------------------------------------------------------------------
OXA-181     -------------------------------------------------------------------------------------------
OXA-204     -------------------------------------------------------------------------------------------
OXA-232     -------------------------------------------------------------------------------------------
OXA-244     -------------------------------------------------------------------------------------------

                   100       110       120       130       140      150           170      180       190
                    *         *         *         *         *        *             *        *         *
OXA-48      VFKWDGQTRDIATWNRDHNLITAMKYSVVPVYQEFARQIGEARMSKMLHAFDYGNEDISGNVDSFWLDGGIRISATEQISFLRKLYHNKLH
OXA-162     -------------------------------------------------------------------------------------------
OXA-163     -------------------------------------------------------------------------------------------
OXA-181     ------------A-----D---------------------------------------------------------Q--A-----------
OXA-204     ------HR-----------------------------------------------------------------------------------
OXA-232     ------------A-----D---------------------------------------------------------Q--A-----------
OXA-244     -------------------------------------------------------------------------------------------

                    200      210        220    230      240       250      260       270
                     *        *          *      *        *         *        *         *
OXA-48      VSERSQRIVKQAMLTEANGDYIIRAKTGYSTRIEPKIGWWVGWVELDDNVWFFAMNMDMPTSDGLGLRQAITKEVLKQEKIIP
OXA-162     ------------------------------A---------------------------------------------------
OXA-163     -----------------------------D-////-----------------------------------------------
OXA-181     ----------------------------------------------------------------------------------
OXA-204     ----------------------------------------------------------------------------------
OXA-232     -------------------------------S--------------------------------------------------
OXA-244     -------------------------------G--------------------------------------------------
```

Figure 1. Amino acid alignment of OXA-48 and six other variants. Dashes indicate identical residues among all amino acid sequences. Slashes indicate the absence of amino acids. Amino acid motifs that are well conserved among class D β-lactamases are indicated by boxes. Numbering is according to class D β-lactamase (DBL nomenclature).[17]

an OD of 0.6 at 600 nm. Then, expression of the β-lactamase genes was carried out overnight at 25°C with 0.1 mM IPTG as inducer. Cultures were centrifuged at 6000 **g** for 15 min and then the pellets were resuspended with 10 mL of 20 mM triethanolamine H_2SO_4 (pH 7.2). Bacterial cells were disrupted by sonication and the bacterial pellet was removed by two consecutive centrifugation steps at 10000 **g** for 1 h at 4°C and then the supernatant was centrifuged at 48000 **g** for 1 h at 4°C. β-Lactamases were purified by using two successive steps of anion-exchange chromatography [20 mM triethanolamine H_2SO_4 (pH 7.2) and then 20 mM piperazine H_2SO_4 (pH 9.5)] using Q-Sepharose columns, followed by a gel filtration step [100 mM sodium phosphate buffer (pH 7) and 150 mM K_2SO_4]. Peaks of β-lactamase activity were concentrated by using Vivaspin® columns (GE Healthcare, Freiburg, Germany) and dialysed with 100 mM sodium phosphate buffer (pH 7). Protein purity was estimated by SDS–PAGE.

Kinetic studies

Purified β-lactamases were used for kinetic measurements, which were determined at 30°C in 100 mM Tris-H_2SO_4 and 300 mM K_2SO_4 (pH 7). The k_{cat} and K_m values were determined by analysing β-lactam hydrolysis under initial-rate conditions with an ULTROSPEC 2000 model UV spectrophotometer (Amersham Pharmacia Biotech) using the Eadie–Hoffstee linearization of the Michaelis–Menten equation. The different β-lactams were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France).

Susceptibility testing

The susceptibility pattern was determined by the disc diffusion method and MICs by the microbroth dilution method. Results were interpreted according to CLSI guidelines.[15] MICs were determined only for clones harbouring recombinant pCR®-Blunt II-TOPO® plasmids and transformed into either *E. coli* TOP10 or *E. coli* HB4 strains.

Results and discussion

Susceptibility patterns of the recombinant strains expressing the OXA-48-like β-lactamases

MIC determination for *E. coli* recombinants always showed high-level resistance to ampicillin and temocillin (Table 1). A significant decreased susceptibility to carbapenems was noticed for all clones (4- to 16-fold increase), with the exception of the clone producing OXA-163 with only a 2-fold increase in MICs of imipenem and doripenem, although the MIC of meropenem remained unchanged. Notably, the recombinant strain producing OXA-181 showed the highest MICs of carbapenems, while almost identical values were obtained for OXA-48, OXA-162 and OXA-204 producers. Lower MICs were obtained for the OXA-232 producer, suggesting lower hydrolytic activity of that variant towards carbapenems (Table 1).

Recombinant strains producing OXA-163 or OXA-232 had lower MICs of temocillin than those producing other OXA-48 derivatives. This result might have a significant impact considering that several screening techniques of OXA-48-like producing isolates now rely on this peculiar property.

As previously noted, the OXA-163 producer was the only OXA-48-like producer exhibiting elevated MICs of broad-spectrum cephalosporins such as cefotaxime and ceftazidime.

Table 1. MICs of β-lactams

β-Lactam	MIC (mg/L)													
	E. coli TOP10 (pTOPO-OXA-48)	*E. coli* TOP10 (pTOPO-OXA-162)	*E. coli* TOP10 (pTOPO-OXA-163)	*E. coli* TOP10 (pTOPO-OXA-181)	*E. coli* TOP10 (pTOPO-OXA-204)	*E. coli* TOP10 (pTOPO-OXA-232)	*E. coli* TOP10	*E. coli* HB4 (pTOPO-OXA-48)	*E. coli* HB4 (pTOPO-OXA-162)	*E. coli* HB4 (pTOPO-OXA-163)	*E. coli* HB4 (pTOPO-OXA-181)	*E. coli* HB4 (pTOPO-OXA-204)	*E. coli* HB4 (pTOPO-OXA-232)	*E. coli* HB4
Ampicillin	>256	>256	>256	>256	>256	>256	8	>256	>256	>256	>256	>256	>256	8
Temocillin	>256	>256	32	>256	256	32	8	>256	>256	64	>256	>256	>256	32
Cefalotin	16	32	64	32	16	64	8	>256	>256	>256	>256	256	>256	64
Cefotaxime	0.25	0.5	16	1	0.25	0.12	0.06	8	8	128	8	4	2	0.5
Ceftazidime	0.25	0.5	64	0.5	0.5	1	0.12	1	1	256	0.5	0.5	0.5	0.5
Imipenem	2	2	0.5	4	2	1	0.25	64	128	0.5	128	64	16	0.25
Meropenem	0.25	0.5	0.06	0.5	0.25	0.25	0.06	64	128	4	128	64	32	0.12
Ertapenem	0.5	0.5	0.06	1	0.5	0.5	0.01	256	>256	32	>256	256	256	1
Doripenem	0.5	0.5	0.12	0.5	0.5	0.25	0.06	32	64	2	32	16	16	<0.03

Impact of the OXA-48-like β-lactamases in a porin-deficient E. coli background

Expression of the bla_{OXA-48}-like genes in *E. coli* HB4 gave rise to higher MICs of carbapenems compared with those MICs obtained for *E. coli* TOP10 (Table 1). Notably, production of OXA-163 increased the MIC of imipenem only slightly although those of other carbapenems, in particular ertapenem, increased more significantly. This result further confirms that OXA-163 is a very weak carbapenemase that does impact the activity of carbapenems only slightly in the absence of additional mechanisms of resistance.

Notably, despite conferring similar resistance patterns, discrepancies among the different OXA-48-like enzymes were highlighted here. For example, MICs of carbapenems were much higher for OXA-181 than for OXA-232 when produced in *E. coli* HB4 (128 versus 16 mg/L) (Table 1).

Hydrolytic properties of OXA-48-like β-lactamases

Kinetic studies were performed with purified OXA-48-like β-lactamases in order to compare their relative catalytic properties. The hydrolysis rates of purified OXA-162, OXA-181 and OXA-204 enzymes for all β-lactams tested were very similar to those obtained with OXA-48 (Table 2). These results are in accordance with MIC data showing very similar susceptibility patterns for the different *E. coli* clones producing OXA-48, OXA-162, OXA-181 and OXA-204, respectively (Table 2). Unlike other variants, OXA-232 had a catalytic activity for temocillin that was ~10-fold lower than that of OXA-48, while hydrolysis of temocillin by OXA-163 remained undetectable. The hydrolysis rates of carbapenems by OXA-232 were significantly lower than those obtained for OXA-48, OXA-162, OXA-181 and OXA-204 (Table 2).

Biochemical analysis of the hydrolytic properties of OXA-163 confirmed that it is an ESBL hydrolysing cefotaxime and ceftazidime efficiently, but carbapenems only marginally.

Conclusions

Several biochemical features of the different OXA-48-like β-lactamases were compared. The results showed that this group of enzymes, although being quite homogeneous in terms of protein sequence, is rather heterogeneous in terms of hydrolytic profile. Indeed, and as opposed to what was initially considered, this group of enzymes does not encompass only carbapenemases. It was shown that subtle amino acid changes may basically confer different substrate specificities, with some variants being either ESBLs or true carbapenemases. Therefore, detection of those genes encoding OXA-48-like enzymes possessing carbapenemase activities only cannot rely on PCR-based amplification of those genes only. Complete sequencing of the corresponding genes is therefore required to extrapolate the kinetic profile of the corresponding enzymes.

In addition, we showed here that additional non-enzymatic resistance mechanisms are required in order to achieve high MIC values of carbapenems for most OXA-48-like producers and that the resistance levels obtained in a porin-deficient *E. coli* background are variable depending on the nature of the OXA-48-like variant.

Additionally, we showed that high-level resistance to temocillin is not a common feature for all OXA-48-like producers.

Table 2. Kinetic parameters of OXA-48 and OXA-48-like β-lactamases

Substrate	K_m (μM)						k_{cat} (s^{-1})						k_{cat}/K_m (mM^{-1}/s^{-1})					
	OXA-48	OXA-162	OXA-163	OXA-181	OXA-204	OXA-232	OXA-48	OXA-162	OXA-163	OXA-181	OXA-204	OXA-232	OXA-48	OXA-162	OXA-163	OXA-181	OXA-204	OXA-232
Benzylpenicillin	ND	35	13	90	90	60	ND	123	23	444	353	125	ND	3400	1800	5000	4100	2100
Ampicillin	400	315	315	170	450	220	955	269	23	218	389	132	2400	830	70	1300	860	600
Oxacillin	95	75	90	80	100	130	130	3	34	90	56	156	1400	40	370	1100	540	1200
Temocillin	45	170	NH	60	75	60	0.3	0.7	NH	0.3	0.5	0.03	6	4	ND	5	7	0.5
Cefalotin	195	180	10	250	270	125	44	12	3	13	12	13	225	70	300	50	45	105
Cefotaxime	>900	310	45	>1000	990	>1000	>9	3	10	>62	12	>6.5	10	10	230	13	12	6
Ceftazidime	NH	NH	>1000	NH	NH	>1000	NH	ND	8	ND	ND	>0.6	NH	ND	3	ND	ND	0.1
Imipenem	13	25	520	13	9	9	5	11	0.03	7.5	4	0.2	370	420	0.06	550	420	20
Meropenem	10	80	>2000	70	60	100	0.07	0.1	>0.1	0.1	0.05	0.03	6	1.3	0.03	1.5	0.8	0.3
Ertapenem	100	30	130	100	90	110	0.13	0.3	0.05	0.2	0.1	0.04	1	9	0.3	2	1	0.4
Doripenem	ND	50	NH	55	25	10	ND	0.05	NH	0.04	0.02	0.005	ND	1	NH	0.7	0.8	0.5

ND, not determined; NH, no detectable hydrolysis was observed with 1 μM purified enzyme and up to 500 μM substrate.
Data are the means of three independent experiments.
Standard deviations were within 10% of the means.
Data for OXA-48 are from Docquier *et al.*[18]

However, resistance to carbapenems correlates well with the carbapenemase activity of these different enzymes. OXA-163 (without any significant carbapenemase activity) and OXA-232 (with weaker carbapenemase activity compared with other OXA-48-like enzymes) hydrolysed temocillin weakly and corresponding producers did not exhibit high MICs of that antibiotic. This suggests that detection and screening strategies based on temocillin resistance are valid for OXA-48-like enzymes with significant carbapenemase activity only. On the other hand, the Carba NP test[16] is an excellent tool for evaluating the carbapenemase activity of these OXA-48-like enzymes.

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Transparency declarations

An international patent form for the Carba NP test has been deposited on behalf of INSERM Transfert (Paris, France).

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