

Full Length Research Paper

Biochemical characterization of a cefotaxime-hydrolysing β -lactamase encoded by a conjugative plasmid

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During the nosocomial infections occurring in the intensive care unit of the military hospital of Tunis in 2005, the *Enterobacter cloacae* BW 1150 strain was isolated from a stool culture. This strain was found to have a high level resistance to broad-spectrum β -lactams. Resistance profile against the various families of antibiotics was determined using the disc diffusion test. The minimal inhibitory concentrations values showed that this strain was resistant to the β -lactams such as ampicillin and the extended spectrum cephalosporins (cefotaxime, ceftriaxon and cefpirome). Analysis of this strain by the disk diffusion test revealed synergies between amoxicillin-clavulanate (AMX-CA) and ceftriaxon, ceftazidime and cefotaxime. Cell sonicate of this isolate is very active against cefotaxime and showed a specific activity (AS) of 7.54 U/mg for the same antibiotic. This activity was inhibited by the sulbactam and the clavulanic acid. Isoelectrofocusing methods revealed that the crude extract of the *E. cloacae* BW 1150 strain showed 1 β -lactamase activity with an isoelectric point (pI) of about 8. This activity was transferred by conjugation and was highly expressed in the transconjugant.

Key words: β -lactams, β -lactamases, cefotaximase, *Enterobacter cloacae*.

INTRODUCTION

Enterobacter species are a common cause of several human diseases and are predominantly associated with nosocomial infections. They are responsible for 5% of all nosocomial septicaemia cases in the United States and can be found as the third most common pathogens recovered from the respiratory tracts of patients in intensive care units (Stock et al., 2001). Among the variety of *Enterobacter* species, *E. cloacae* is a well-recognized nosocomial pathogen that causes significant infections, especially in last recent years (Conceição et al., 2004). This microorganism is the most commonly isolated member of the *Enterobacteriaceae* that possess a chromosomally encoded ampC β -lactamase that plays

an important role in resistance to antibiotics (Bell et al., 2003). However, several reports have demonstrated that this species can acquire and express genes encoding extended spectrum β -lactamase (ESBLs), especially for the multidrug isolates (Hall et al., 2006). In fact, it has been reported that the wild-type strains of *E. cloacae* can express the bush group 2f carbapenemases Nmc-A, VIM-2, IMI-2 and VIM-4 (Pottumarthy et al., 2003; Jeong et al., 2003; Yu et al., 2006; Luzzaro et al., 2004). The resistance to broad-spectrum penicillins and extended-spectrum cephalosporins via the acquisition of plasmids encoding TEM and SHV derivatives BLSEs has also been reported (Liu et al., 2004). Various extended-spectrum β -lactamases not derived from TEM or SHV enzymes have been described in this species, such as SFO type in Japan (Matsumoto et al., 1999), IBC-1 type in Greece (Giakkoupi et al., 2000), VEB-3 type in Thailand (Jiang et al., 2005) and CTX-M class that have been des-

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cribed more recently (Doucet et al., 2000; Chanawong et al., 2002; Kim et al., 2005; Touati et al., 2006).

In this work, we report the biochemical analysis of cefotaximase producing clinical isolate of *E. cloacae* from the military hospital of Tunis.

MATERIALS AND METHODS

Bacterial strains

One isolate of *E. cloacae* BW 1150 is included in this study. This strain was isolated from a stool culture in intensive care unit of the military hospital of Tunis in 2005. This isolate was identified using the API 20 E identification system (bioMérieux, Marcy l'Etoile, France). *E. coli* HB101 (F⁻, Δ(gpt-proA) 62, leuB6, supE44, ara-14, galK2, lac Y1, Δ(mcrC-mrr), rps, L26, Xyl-rmt1 1, thi-1, IncFI, rec AB, str^r) is resistant to streptomycin and is used for the conjugation experiences.

Susceptibility testing and extended-spectrum β-lactamase detection

The antibiotics susceptibilities of the *E. cloacae* BW 1150 strain was determined on Mueller-Hinton agar by the standard disk diffusion procedure as described by the antibiogram committee of the French society for microbiology (www.sfm.asso.fr) (Cavallo et al., 2007). The following antibiotics were tested, ampicillin, ticarcillin, cefoxitin, cefotaxime, ceftazidime, ceftriaxone, ceftiofime, streptomycin, ciprofloxacin, aztreonam, imipenem, tetracycline, chloramphenicol, acid nalidixique (Biorad, Marnes-la-Coquette, France).

The ESBL detection was based on the double-disk synergy test (DDST) as described previously (Ben et al., 2007). DDST was performed as follows: the surface of a Mueller-Hinton (MH) agar plate was inoculated with an overnight culture suspension of clinical isolate. After inoculation, disks containing 30 μg of ceftazidime, cefotaxime, ceftriaxone, ceftiofime and amoxicillin-clavulanic acid (20/10 μg) were placed at distances of 20 mm (center to center). Enhancement of the inhibition zone between the disks containing clavulanic acid and cefotaxime, ceftriaxone, ceftazidime or ceftiofime indicated the presence of ESBL production.

Determination of minimum inhibitory concentration

Minimum inhibitory antibiotic concentrations were determined by the serial dilution method. The minimum inhibitory concentration results were interpreted according to the recommendations of the antibiogram committee of the French society for microbiology (ACFSM) (Cavallo et al., 2007).

Preparation of crude enzyme extracts

Bacterial strain was grown overnight with shaking in 50 ml of trypticase soy broth (TSB) (Diagnostics, Pasteur, France) at 37°C. The cells were harvested by centrifugation and washed once in 25 mM potassium-sodium phosphate buffer (pH 7) and resuspended in 1 ml of the same buffer.

For preparation of cell-free extract, the cells were ruptured by ultrasonic treatment in a UP 400 S sonicator at 4°C. Cell debris was removed by centrifugation at 10000 rpm for 10 min in a Hettich centrifuge R 32 rotor. The supernatant was used for all β-lactamases enzyme tests (Ben et al., 2007).

β-Lactamase assay

β-Lactamase activities were determined by spectrophotometric method of O'calloghan et al. (1975). Briefly, the decrease in absorbance of the antibiotics at an appropriate concentration and wavelength was measured in a temperature controlled spectrophotometer (Varian[®] CARY 50 Bio UV-visible) at 37°C (Ross et al., 1975).

Inhibitors effects (IC₅₀ determination)

For determination of inhibitor effects, rates of hydrolysis of 1 mM cephalotin were determined in the presence of various concentrations of clavulanic acid and sulbactam. EDTA (Ethylenediamine tetraacetic acid) was used for 1 mM as concentration. In these experiments the proteic extract was preincubated with the inhibitors for 10 min before the addition of cephalotin. The inhibitory concentration that allowed the reduction of β-lactamases activities of 50% was determined from the curve: AS = f (inhibitors concentration) (Réjiba et al., 2002).

Conjugation and resistance transfer

Conjugation experiment using *Escherichia coli* HB101 str^R as the recipient strain was performed. Transconjugants were selected on Miller-Hinton agar plates containing streptomycin (20 μg/ml), ticarcillin (2 μg/ml) and cefotaxime (2 μg/ml). To select the plasmid-encoded resistance, the transjugants growing on the selection plates were subjected to DDST to confirm the presence of ESBL (Ben-Hammouda et al., 2004).

Analytical isoelectric focusing (IEF)

IEF was performed as follows, bacterium growing exponentially at 37°C in trypticase soy broth was harvested at 10000 rpm for 10 min (Beckman centrifuge, FO650 Rotor) and cell-free lysate was prepared by sonication (sonicator UP 400S cycle: 25 and amplitude 50 Hz). Crude sonic extract was centrifuged at 10000 rpm for 10 min and the supernatant was collected and subjected to IEF in broad range (Ph 3 - 10) polyacrylamide gel 7% at 4°C. The β-lactamase activity was detected by the iodine procedure in gel by using benzylpenicillin (30 μg/ml) as the substrate (Ross et al., 1975).

RESULTS

Ssusceptibility testing and extended-spectrum β-lactamase detection

Antibiotics susceptibility testing revealed that this isolate was resistant to all penicillins and cephalosporins tested except ceftazidime. For its resistance to others antibiotics, this strain was resistant to chloramphenicol, tetracycline, nalidixic acid. Whereas, this isolate remain susceptible to imipenem and streptomycin (Table 1). The enhancement of the inhibition zone between the disks containing amoxicillin-clavulanate and cefotaxime, ceftriaxone and ceftiofime indicated the presence of ESBL production (data not shown).

Minimal inhibitory concentration

The resistance to β-lactams of *E. cloacae* BW 1150 strain

Table 1. Antibiotics susceptibility of the *E. cloacae* BW 1150 strain, transconjugant and *E. coli* recipient.

Antibiotic	<i>E. cloacae</i> BW 1150	<i>E. coli</i> HB 101 x <i>E. cloacae</i> BW 1150	<i>E. coli</i> HB 101
Ceftazidime	22	20	25
Cefotaxime	12	14	26
Cefpirome	13	14	26
Ceftreaxone	14	14	23
Streptomycin	27	14	14
Ciproflaxacin	15	13	28
Ticarcillin	6	6	25
Nalidixic acid	8	6	30
Ampicillin	6	6	32
Aztreonam	18	16	35
Cefoxitim	6	28	32
Cloramphenicol	6	24	34
Tetracyclin	6	24	29
Imipinem	25	32	35

Table 2. MIC values ($\mu\text{g/ml}$) of the *E. cloacae* BW 1150 strain, transconjugant and *E. coli* recipient.

Antibiotic	<i>E. cloacae</i> BW 1150	<i>E. coli</i> HB101x <i>E. cloacae</i> BW 1150	<i>E. coli</i> HB 101
Ceftazidime	8	4	<1
Céfotaxime	>512	>512	1
Cefpirome	>512	>512	2
Ceftreaxone	>512	>512	<1
Streptomycin	4	>256	>256
Ciproflaxacin	>512	>256	2
Ticarcillin	>512	>256	2
Nalidixic acid	512	>256	1
Ampicillin	>512	>256	4
Aztreonam	128	128	4
Cefoxitim	512	4	2
Cloramphénicol	256	4	<1
Tetracyclin	512	2	2
Imipinem	4	4	1

is confirmed by the Minimal inhibitory concentration of the penicillins (ampicillin and ticarcillin), the extended spectrum cephalosporins (cefoxitin, cefotaxime, ceftriaxone, cefpirom except ceftazidime) which exceed 256 $\mu\text{g/ml}$. This strain is also resistant to others antibiotic, in addition to the β - lactams. Indeed, this strain is highly resistant to cloramphenicol, tetracyclin and quinolones (Nalidixic acid) with a value that exceed 256 $\mu\text{g/ml}$. Nevertheless, it remains sensitive to imipinem and streptomycin (Table 2).

β -Lactamase assay and inhibitor susceptibility

Analysis of the crude extract by the starch, 7% polyacrylamide gel electrophoresis revealed one band in the presence of benzylpenicillin; the same band was visualized with cefotaxime (Figure 1).

E. cloacae BW 1150 hydrolyzed oxyimino-cephalosporins in addition to benzylpenicilline and ticarcillin. Cefotaxime was the most stable among the cephalosporins tested. The hydrolysis of imipinem cloxacillin and

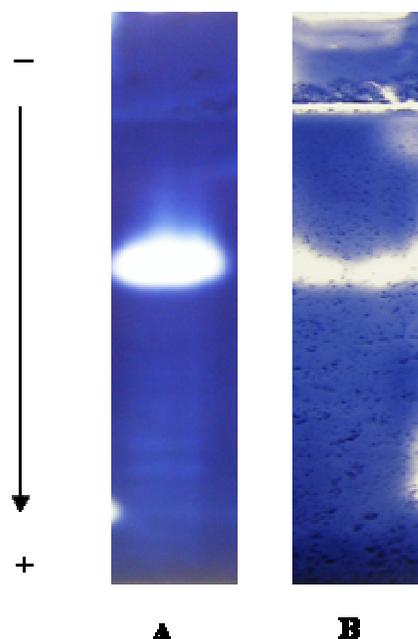


Figure 1. Activity β -lactamase detection of the *E. cloacae* BW 1150 strain after native electrophoresis 7.5% in the presence of 1 mM benzylpenicillin (A) and 3 mM cefotaxime (B) as substrates.

Table 3. β -Lactamases specific activity (μ mole of substrat/minut/mg of protein) produced by *E. cloacae* BW 1150.

Antibiotic	AS (U/mg) <i>E. cloacae</i> BW 1150
Benzylpenicillin	0.91
Ticarcillin	0.34
Cefoxitin	1.82
Cefotaxime	7.54
Ceftazidime	0.11
Aztreonam	0.25
Imipinem	ND
Cloxacillin	ND
Cefalotin	1.68
Ceftriaxon	0.38

aztreonam by *E. cloacae* BW 1150 was not detectable (Table 3). *E. cloacae* BW 1150 activity was inhibited by clavulanic acid and sulbactam but was not inhibited by EDTA (Table 4). These characteristics resembled those of class A enzymes reported previously (Bush et al., 1995).

Conjugation and resistance transfer

Conjugation from *E. cloacae* BW 1150 to *E. coli* HB 101 was successful. Only the resistance to ampicillin,

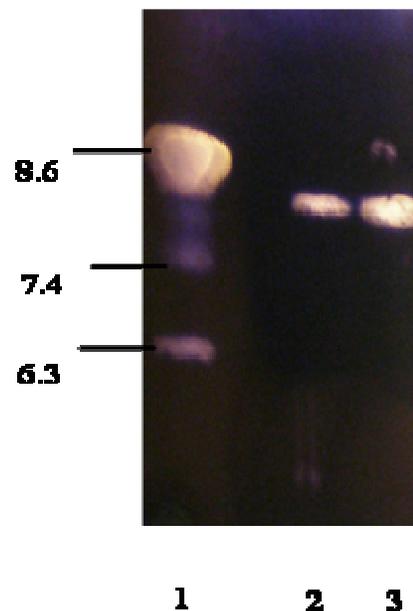


Figure 2. Isoelectric focusing of *E. cloacae* BW 1150 and its transconjugants. Lane 1, Markers; lane 2, *E. cloacae* BW 1150; lane 3, *E. cloacae* BW 1150 x *E. coli* HB 101.

ticarcillin, cefotaxime and ceftazidime was transferred, suggesting that the genes of resistance to these antibiotics are on a conjugable R plasmid (Table 1). This resistance is confirmed by the minimal inhibitory concentration (Table 2). The enhancement of the inhibition zone between the disks containing clavulanic acid and cefotaxime and ceftazidime indicated the presence of ESBL production (data not shown).

Analytical isoelectric focusing (IEF)

The supernatants of *E. cloacae* BW 1150 and the transconjugant were collected and subjected to IEF in broad range (pH 3 - 10) polyacrylamide gel 7% at 4°C. The β -lactamase activity was detected according to the iodometric method in the presence of the benzylpenicillin as substrate. The *E. cloacae* BW 1150 and its transconjugant produce the same β -lactamase activity with a pI of about 8 (Figure 2).

DISCUSSION

The clinical isolate of *E. cloacae* BW 1150 included in this study was isolated in the intensive care unit at the military hospital in Tunisia in 2005. This isolate showed a multi-drug resistance phenotype including resistance to the extended spectrum β -lactams except the ceftazidime, cloramphenicol, tetracyclin and quinolone (Nalidixic acid). Whereas, this isolate was found susceptible to imipinem

Table 4. Inhibitor effects on β -lactamase activity produced by *E. cloacae* BW 1150.

Organism	(IC ₅₀ μ M)		
	Clavulanic acid	Sulbactam	EDTA
<i>E. cloacae</i> BW 1150	0.76	1.34	-

(-) without effect.

the ICM of various antibiotics was determined, which showed a high level of resistance for this isolate. The double disk synergy gave a positive result for this isolate, suggesting the presence of extended spectrum- β -lactamase. This activity was strongly active against cefotaxime and it was inhibited by the clavulanic acid which confirm that it belong to serine active β -lactamase. Conjugation experiment using *E. coli* HB 101 S^R as the recipient strain was performed. Penicillines and cephalosporines resistance was transferred by conjugation. Isoelectric focusing analysis of the supernatant of *E. cloacae* BW 1150 and its transconjugant showed 1 band with pI of about 8. This result showed that this enzyme is on a conjugable plasmid. According to the β -lactam susceptibility profile, the specific activity and the isoelectric point, this enzyme seems to have resemblances with other CTX-M-type β -lactamases (Bonnet et al., 2004). Indeed, this β -lactamase hydrolyze all ampicillins and cephalosporins with a high hydrolytic activity to cefotaxime. Furthermore, the specific activity of this isolate was restored in the presence of inhibitors. Considering its isoelectric point, the β -lactamase of pI of about 8 is similar to CTX-M-9 previously described (Sabate et al., 2000; Chanawong et al., 2002).

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