

Full Length Research Paper

# High-level cefotaxime-resistant *Proteus mirabilis* strain isolated from a Tunisian intensive care unit ward: CTX-M-8 extended-spectrum $\beta$ -lactamase coproduced with a plasmid mediated AmpC lactamase

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The aimed of this study was to determine the implication of the biochemical and the molecular mechanism and to describe the properties of an extended-spectrum  $\beta$ -lactamase (ESBL) CTX-M-8 which was reported for the first time in Africa. A clinical isolate of *Proteus mirabilis* FS6449 was isolated from a patient hospitalized at an intensive care unit of the Military Hospital in Tunisia in 2009. Antimicrobial susceptibility was determined with the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines and revealed that this strain was resistant to expanded-spectrum  $\beta$ -lactams. Analysis of *P. mirabilis* FS6449 by double-disk synergy test yielded a positive result suggesting the production of ESBLs. Sonicate of the isolate hydrolysed cefotaxime and benzylpenicillin. Isoelectric focusing exhibited four  $\beta$ -lactamase bands of isoelectric points (pIs) 5.6, 6, 6.5 and over 7.6. Polymerase chain reaction (PCR) and sequencing experiments revealed the presence of four  $\beta$ -lactamase genes encoding TEM-2, CTX-M-8, TEM-24, and an AmpC enzyme. Among them, the genes encoding TEM-24 and an AmpC enzyme were transferred to the recipient by conjugation experiments.

**Key words:** Resistance,  $\beta$ -lactamase, *Proteus mirabilis*.

## INTRODUCTION

Plasmid-mediated extended-spectrum  $\beta$ -lactamases (ESBLs) capable of degrading the expanded-spectrum cephalosporins and monobactams are the most important resistance determinants emerging worldwide in Enterobacteriaceae (Mugnaioli et al., 2006). Since the late 1990s, Enterobacteriaceae producing CTX-M-type typical

ESBLs and have therefore been classified as the functional group 2be of the Bush-Jacoby-Medeiros classification scheme (Touati et al., 2006). The CTX-M-type  $\beta$ -lactamases, encoded by genes that have been captured on transferable plasmids from the chromosomes of *Kluyvera* spp., are the most common and widespread ESBLs encountered in Enterobacteriaceae (Mugnaioli et al., 2006). At present, the CTX-M family comprises more than 60 enzymes ([www.lahey.org/studies/webt.asp](http://www.lahey.org/studies/webt.asp)) and can be sub-classified by amino acid sequence similarities into five major phylogenetic groups: the CTX-M-1 group including CTX-M-1, -3, -10, -11, -12, -15, -22, -23, -28, -29, -30, -32, -33, -36, -54 and UOE-1; the CTX-M-2 group including CTX-M-2, -4, -6, -7, -20, -31, -44 (previously Toho-1) and FEC-1; the CTX-M-8 group including CTX-M-8, -40 and -63; the CTX-M-9 group including CTX-M-9, -13, -14, -18, -16,

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**Abbreviations:** MICs, Minimum inhibitory concentrations; CLSI, Clinical and Laboratory Standards Institute.

ESBLs have emerged in hospital and community settings as important sources of urinary tract infections (UTIs) (Minarini et al., 2009). CTX-M-type  $\beta$ -lactamases are

-17, -19, -21, -24, -27, -45 (previously Toho-2), -46, -47, -48, -49 and -50; the CTX-M-25 group with CTX-M-25, -26, -39 and -41 (Ben Achour et al., 2009a; Wu et al., 2006; Bourouis et al., 2009). The group CTX-M-8 is the rarest CTX-M subgroup, with only one previously identified member, CTX-M-8 (Bonnet et al., 2000). In Tunisia, the prevalence of ESBLs in clinical isolates of Enterobacteriaceae varies from 20% (TEM) to 45% (CTX-M) and 35% (SHV). The SHV-2 enzyme was the first ESBL to be isolated, in 1984 from a *Klebsiella pneumoniae* strain in Tunisia. Recently, SHV-12, SHV-2a, CTX-M-3, CTX-M-27, CTX-M-28, CTX-M-15, CTX-M-16 and CTX-M-9 have been described in Tunisians hospitals (Armand-Lefevre et al., 2003; Elhani et al., 2010; Bourouis et al., 2009). Several reports have detected TEM-type ESBL family such as TEM-4, TEM-15, TEM-138 and TEM-164 (Ben Achour et al., 2009b). SHV-11 (Ben Achour et al., 2008), SHV-104 (EU032604), SHV-28 (Dahmen et al., 2010), SHV-27 (Abassi et al., 2008) were recently reported in *K. pneumoniae* which represents one of the most important pathogens causing nosocomial infections.

Moreover, ESBLs have also been reported in *Escherichia coli* strains of food origin including CTX-M-1, CTX-M-8, CTX-M-14 and SHV-5 (Jouini et al., 2007). *Proteus mirabilis* causes clinically significant infections and is often difficult to eradicate from the host (De champs et al., 2000). Various ESBLs, such as TEM-, CTX-M-, PER-, and VEB-type ESBLs, have been described in *P. mirabilis* in separate geographic areas (Wu et al., 2008). In the present study, we reported the biochemical and the molecular characteristics of a cefotaxime-resistant *P. mirabilis* strain which coproduced CTX-M-8-type ESBL and AmpC  $\beta$ -lactamase for the first time in Tunisia and Africa.

## MATERIALS AND METHODS

### Bacterial isolates

From June, 2005 to July, 2009, a total of 150 strains of *P. mirabilis* were isolated from patients at the Military Hospital in Tunisia. *P. mirabilis* strain FS6449 was isolated from an intensive care unit on May, 2009. The isolate was identified using a Vitek GNI card (bioMérieux, Marcy l'Etoile, France). *E. coli* DH5 $\alpha$  (recA1, F', endA1, gyrA96, thi-1, hsdR17, rK', mK+, supE44, relA1, DlacU69, F80lazDM15) and nalidixic acid resistant *E. coli* K12 were used respectively for the transformation and the conjugation experiments.

### Antimicrobial susceptibility and synergy testing

Susceptibilities to various antimicrobial agents were tested, and potential ESBL-producing isolates were confirmed by the disk diffusion method on Mueller-Hinton agar (MH; Diagnostics Pasteur, Marcy l'Etoile, France) (Mahrouki et al., 2009). Antibiotic-containing disks were purchased from Bio-Rad, Marnes La Coquette, France. The double-disk synergy test for confirmation of ESBL activity was carried out as described previously (Jarlier et al., 1988), by using disks containing amoxicillin-clavulanate (AMX-CA) against cefotaxime, ceftriaxone and ceftazidime. The minimum inhibitory con-

centrations (MICs) of various  $\beta$ -lactam agents were determined by dilution method on MH agar according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2007).

### Analytical isoelectric focusing (IEF)

The ESBL-producing *P. mirabilis* isolate was subjected to IEF as described previously (Bourouis et al., 2010). Crude extracts were prepared by sonication, and IEF of the enzyme preparations was performed in polyacrylamide gels containing ampholines with a pH range of 3 to 10 using a 111 Mini IEF Cell (Bio-Rad). Extracts from strains producing TEM-1, TEM-2, TEM-3, and SHV-1 were used as standards for pI value of 5.4, 5.6, 6.3, and 7.6, respectively.  $\beta$ -Lactamases were revealed with the iodine method (Mahrouki et al., 2009) with benzylpenicillin (0.5 mM) and cefotaxime (3 mM) in phosphate buffer (25 mM, pH 7).

### $\beta$ -Lactamase assay

Hydrolytic activities of crude extracts for  $\beta$ -lactam antibiotics were determined by the spectrophotometric method at the wavelength of maximal absorbance for the  $\beta$ -lactam ring of each antibiotic (Philippon et al., 1997). The decrease in absorbance of the antibiotics at an appropriate concentration was measured in a temperature controlled spectrophotometer (Varian R CARY 50 Bio UV- visible) at 37°C. Specific activity is calculated on depending of Ross and O'Callaghan equation in 1975 (Ross et al., 1975).

### Polymerase chain reaction (PCR) amplification and DNA sequencing

The presence of *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub> and *bla*<sub>AMPC</sub> were detected by PCR and nucleotide sequencing with appropriated oligonucleotide primers (Table 1). Four primer sets were used in reactions with plasmid preparations from *P. mirabilis* FS6449 to amplify *bla*<sub>CTX-M</sub> sequences and were designated CTX-M-1, CTX-M-2, CTX-M-8 and CTX-M-9 (Ben Achour et al., 2009a). Nucleotide and amino acid sequence analyses were performed using NCBI analysis tools. The resulting sequences were then compared with the reported sequences from GenBank at <http://www.lahey.org/studies/webt.html>.

### Plasmid DNA isolation and transfer of resistance

Plasmid DNA was extracted from the clinical isolate and transconjugant by the alkaline lysis method described by Kado and Lui (1981). DNA electrophoresis was performed in 0.8% agarose. Gels were stained with ethidium bromide and photographed using Polaroid film with a ultraviolet (UV) light source. Transformation experiments were carried out by using *E. coli* DH5 $\alpha$  as the recipient. Transformants were selected on Luria-Bertani medium agar plates supplemented with ampicillin (100 mg/L).

### Conjugation experiments

Conjugation experiments were performed by a liquid mating method on Luria-Bertani broth medium as previously described (Ben Hamouda et al., 2004). Culture mixtures were incubated overnight at 37°C with FS6449 strain as donor and *E. coli* K12 as the recipient at a 1:2 ratio (donor to recipient). Transconjugants were selected on Luria-Bertani broth agar plates supplemented by 256  $\mu$ g of nalidixic acid and 100  $\mu$ g of ampicillin per millilitre. The resulting transconjugants were subjected to antimicrobials susceptibility and isoelectric focusing analysis.

**Table 1.** Sequences of the primers used to detect *bla*- genes.

Target	Primer name	Primer sequence	Annealing temperature (°C)	Amplicons size (bp:base pairs)
CTX-M-1 group	CTXM-1F	5'ATGGTTAAAAAATCACTGCGTC 3'	60	864
	CTXM-1R	5'TTGGTGACGATTTTAGCCGC 3'		
CTX-M-2 group	CTXM-2F	5'ATGATGACTCAGAGCATTTCG3'	58	866
	CTXM-2R	5'TGGGTTACGATTTTCGCCGC3'		
CTX-M-8 group	CTX-M-8F	5'ACTTCAGCCACACGGATTCA3'	60	877
	CTX-M-8R	5'CGAGTACGTCACGACGACTT3'		
CTX-M-9 group	CTXM-9F	5'ATGGTGACAAAGAGAGTGCAA 3'	60	876
	CTXM-9R	5'TCACAGCCCTTCGGCGATGATTCTCGC 3'		
AmpC	AmpC 1	5'ATGATGAAAAAATCGTTATGC 3'	64	1,143
	AmpC 2	5'TTGCAGCTTTTCAAGAATGCGC 3'		

**Table 2.** MICs of various antimicrobial agents obtained for the clinical isolate *P. mirabilis* FS6449, transformants, transconjugants and the *E. coli* recipients.

Antibiotic	MIC (mg/L)				
	<i>P. mirabilis</i> FS6449	<i>E. coli</i> K12x pFS6449	<i>E. coli</i> K12	<i>E. coli</i> DH5α/pFS FS6449	<i>E. coli</i> DH5α
Ampicillin	>512	512	8	512	8
Ticarcilline	>512	>512	8	>512	2
cephalothin	>512	512	<2	512	2
Cefoxitin	32	128	2	64	4
Cefotaxime	512	512	<2	512	<2
Ceftriaxone	512	512	<2	512	<2
Ceftazidime	>512	512	<2	512	<2
Aztreonam	256	128	<2	64	<2
Nalidixic acid	64	>512	512	8	<2
Ciprofloxacin	4	4	2	4	<2
Chloramphenicol	256	8	<2	8	2
Tetracycline	128	8	<2	4	<2
Oflaxacin	256	4	2	2	<2
Streptomycin	512	256	2	256	<2
Imipenem	4	2	<2	2	<2

## RESULTS

*P. mirabilis* FS6449 was isolated in 6 May, 2009 from a blood specimen of an inpatient. Antimicrobial susceptibility testing showed that this isolate presented a multi-drug resistance phenotype including resistance to the extended-spectrum  $\beta$ -lactams (Table 2). The double-disk synergy tests showed positive results for the wild strain, its transformants and transconjugants, suggesting the presence of a class A ESBL. The isolate exhibited a high level resistance not only to cefotaxime (MIC, 512 mg/L), ceftazidime (>MIC, 512 mg/L), and aztreonam (MIC, 512 mg/L) but also to cefoxitin (MIC, 32 mg/L). The strain was also resistant to tetracycline, chloramphenicol, amikacin, gentamycin, and streptomycin (Table 2). Similar results were observed with the transformant and transconjugant

except for chloramphenicol and tetracycline. Analytical isoelectric focusing with benzylpenicillin as substrate of crude  $\beta$ -lactamases extract of *P. mirabilis* FS6449 revealed that this strain produces four  $\beta$ -lactamases with pIs of 5.6, 6, 6.5 and over 7.6. Only the  $\beta$ -lactamases of pI 6.5 and over 7.6 were detected on the crude  $\beta$ -lactamases extract of transformant and transconjugant. The resistance to expanded-spectrum cephalosporins was successfully transferred to a recipient strains, *E. coli* K12 with the frequency of transfer equivalent to  $7.2 \times 10^{-2}$  transconjugant/recipient and *E. coli* DH5 $\alpha$ . This resistance was confirmed by the MICs (Table 2). The results of plasmid analysis showed that the transconjugant and the transformant have similar profiles compared-to-the-parental strain. PCR- and-sequencing experiments specific for the *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub>

**Table 3.** Specific activities  $\beta$ -lactamases of *P. mirabilis* strain FS6449 ( $\mu\text{mol}$  of substrate hydrolyzed/min/mg of protein).

Antibiotic	FS6449
Benzylpenicillin	6.95
Ticarcillin	5.87
Imipenem	ND
Cephalotin	1.76
Cefotaxime	5.45
Ceftriaxone	0.89
Ceftazidime	0.67
Aztreonam	0.20
Cefpirome	0.45
Oxacillin	1.20

ND, Non detected.

genes showed the detection of CTX-M-8 and TEM-2 and confirmed the presence of TEM-24 in parental strain FS6449 and its transconjugant *E. coli* K12x pFS6449 and transformant *E. coli* DH5 $\alpha$ /pFS FS6449. The PCR with the consensus primers of *Citrobacter freundii ampC* family as previously described (Winkor et al., 2001) was positive in parental strain and the transconjugants and electroporant which show the coproduction of a plasmid-mediated AmpC type cephalosporinase with the CTX-M-8. FS6449 *P. mirabilis* strain have showed a high level of hydrolytic activity to benzylpenicillin and ticarcillin, comparable to that observed for the extended-spectrum cephalosporins and the specific activity ranged from 0.20 to 6.95 U/mg of protein. The specific activity values of cephalosporin indicated that cefotaxime is more efficiently hydrolysed than ceftazidime and ceftriaxone (Table 3).

## DISCUSSION

Plasmid-encoded ESBLs impair the efficacy of expanded-spectrum cephalosporins and monobactams and are among the most important acquired resistance determinants emerging worldwide in members of the Enterobacteriaceae (Bradford, 2001; Paterson and Bonomo, 2005; Bonnet, 2004). In recent years CTX-M enzymes have become the most widespread ESBLs and they confer a high level resistance to cefotaxime but have a low level activity towards ceftazidime (Canton and Coque, 2006; Eckert et al., 2004). In Tunisia the frequency of acquired resistance to broad-spectrum cephalosporins seemed to be increasing the last years. In the region of Sfax (Tunisia), the overall frequency of resistance increased from 10% in 1999 to 18% in 2005 (Mkaour et al., 2008). This increase was statistically significant. High prevalence rates of third generation cephalosporins resistance have been observed in intensive care units (48%), hematology and oncology wards

(27%) and pediatric wards (25%) (Mkaour et al., 2008). In the present study, we describe the presence of a CTX-M-8-type ESBL coproduced with a plasmid-mediated AmpC  $\beta$ -lactamase in a clinical isolate for the first time in Tunisia.

The phenotypic study via the susceptibility test and the minimum inhibitory concentration determination showed that *P. mirabilis* FS6449 is a multi-drug resistant isolate and that the transformant and the transconjugant had the same levels of resistance almost. PCR and Isoelectric focusing of strain FS6449 with benzylpenicillin as substrate revealed the presence of four  $\beta$ -lactamases with approximate pI of 5.6; 6; 6.5 and superior than 7.6 which matched respectively to TEM-2, CTX-M-8, TEM-24 and AMPC.

These  $\beta$ -lactamases were active against benzylpenicillin and having a level hydrolytic activity to extended spectrum cephalosporin particularly cefotaxime and ceftriaxone (Table 3). Only  $\beta$ -lactamases with pI of 6.5 and superior than 7.6 were detected on the crude enzyme extract of transformant of *P. mirabilis* FS6449 isolate. This finding indicates that the genetic determinant of CTX-M-8 and TEM-2 may be located on plasmid chromosome. Sequencing experiments showed that the *bla*<sub>CTX-M</sub> gene encoding for CTX-M type  $\beta$ -lactamase was identical to the *bla*<sub>CTX-M-8</sub>.

Few research reports the presence of CTX-M-8 subgroups in Tunisia and the extended-spectrum  $\beta$ -lactamase CTX-M-8 was isolated for the first time in *E. coli* strains of food (chicken) origin. The strain was isolated in 2006 and the phenotypic study showed a high level resistance to tetracycline and nalidixic acid (Jouini et al., 2007). In Tunisia, CMY-4, an AmpC-type plasmid-mediated  $\beta$ -lactamase was the first  $\beta$ -lactamase described in clinical isolate of *P. mirabilis* in January 1996 at Charles Nicole Hospital in Tunis (Verdet et al., 1998) and in 1999, a novel ACC-1, a plasmid-encoded cephalosporinase ACC-1 was identified in clinical isolate of *P. mirabilis* at CHU Habib Bourguiba Hospital in Sfax (Rhimi-Mahjoubi et al., 2002).

The susceptibility testing to cefoxitin show the high level of resistance to the *E. coli* transconjugants and the transformants compared with the parental strain (Table 2). IEF and PCR experiments show that this resistance was due to the production of the plasmid-encoded cephalosporinase which was detected on pI superior than 7.6. This proves the association of hyperproduction of a plasmidic AmpC with CTX-M-8 which exhibit notable activity against ceftazidime, cefotaxime and cefoxitin. The gene *bla*<sub>AMPC</sub> was transferred with *bla*<sub>TEM-24</sub> by conjugation and transformation experiments and they were located on the same large plasmid.

In conclusion, the presence of strains producing CTX-M genes with an association of a plasmidic AmpC in the hospital environment could be linked to insufficient measures in room's surface cleaning, so continued surveillance is essential to control the spread of this resistance.

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