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Genetic and Kinetic Characterization of the Novel AmpC β-Lactamases DHA-6 and DHA-7

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During a Spanish surveillance study, two natural variants of DHA β-lactamases, DHA-6 and DHA-7, were found, with the replacements Ala226Thr and Phe322Ser, respectively, with respect to DHA-1. The DHA-6 and DHA-7 enzymes were isolated from *Escherichia coli* and *Enterobacter cloacae* clinical isolates, respectively. The aim of this study was to genetically, microbiologically, and biochemically characterize the DHA-6 and DHA-7 β-lactamases. The bla_{DHA-6} and bla_{DHA-7} genes were located in the I1 and HI2 incompatibility group plasmids of 87.3 and 310.4 kb, respectively. The genetic contexts of bla_{DHA-6} and bla_{DHA-7} were similar to that already described for the bla_{DHA-1} gene and included the *qnrB4* and *aadA* genes. The MICs for cephalothin, aztreonam, cefotaxime, and ceftazidime were 8- to 32-fold lower for DHA-6 than for DHA-1 or DHA-7 expressed in the same isogenic *E. coli* TG1 strain. Interestingly, the MIC for cefoxitin was higher in the DHA-6-expressing transformant than in DHA-1 or DHA-7. Biochemical studies with pure β-lactamases revealed slightly lower catalytic efficiencies of DHA-6 against cephalothin, ceftazidime, and cefotaxime than those of DHA-1 and DHA-7. To understand this behavior, stability experiments were carried out and showed that the DHA-6 protein displayed significantly higher stability than the DHA-1 and DHA-7 enzymes. The proximity of Thr226 to the N terminus in the tertiary protein structure in DHA-6 may promote this stabilization and, consequently, may induce a slight reduction in the dynamic of this enzyme that primarily affects the hydrolysis of some of the bulkiest antibiotics.

Plasmid AmpC β-lactamases are clinically important cephalosporinases, particularly in *Enterobacteriaceae*, and the transmission of plasmids carrying AmpC genes has been detected in bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* (1, 2). At present, none of the commercially available β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam) inactivate high-level class C producers (3), although tazobactam has shown some inhibitory activities in certain species, such as *Morganella morganii* (4, 5). Boronic acids and other compounds are promising new candidates as AmpC β-lactamase inhibitors (6, 7).

New extended-spectrum class C enzymes that are capable of hydrolyzing imipenem and cephalosporins with large side chains are emerging (2, 8, 9). These enzymes differ from typical AmpC β-lactamases as a result of amino acid insertions, deletions, and substitutions (2, 8). The three regions involved in these modifications are the omega loop, the R2 loop, and the helix H2 (2, 8).

The DHA enzymes are plasmid AmpC β-lactamases that were first described in 1997. Although at least 11 variants have been identified (see http://www.lahey.org/studies), complete kinetic and structural data are not yet available for these enzymes. A comparative study of the carbapenem-hydrolyzing activities of five plasmid-borne AmpC β-lactamases produced some kinetic data on the DHA-1 enzyme for cephaloridine and imipenem only (9), which were not tested in the present work.

The genetic contexts of the DHA-1 β-lactamase gene in different strains of *M. morganii* and *K. pneumoniae* were characterized and found to be similar to each other and included the *qnrB4* (quinolone resistance) and *aadA* (streptomycin and spectinomycin resistance) genes (10, 11, 12).

In this study, we compared the genetic contexts of bla_{DHA-1}, bla_{DHA-6}, and bla_{DHA-7} in *Enterobacteriaceae* clinical isolates from Spain and obtained results that were highly consistent with those of previous work. We also provide here the first kinetic characterization of the DHA-1, DHA-6, and DHA-7 proteins, which differ from each other in two amino acids. By modeling, we concluded that the Thr226 mutation may affect the hydrolysis of some cephalosporins, including extended-spectrum cephalosporins, in DHA-type β-lactamases.

MATERIALS AND METHODS

Antibiotics and other chemicals. Ampicillin, cephalothin, cefoxitin, ceftazidime, cefotaxime, aztreonam, clavulanic acid, sulbactam, and tazobactam were purchased from Sigma Chemical Co. (St. Louis, MO). Cefepime was obtained from Bristol-Myers Squibb (New York, NY). Imipenem and clavulanic acid were gifts from Merck (Whitehouse Station, NJ) and GlaxoSmithKline (Brentford, London, United Kingdom), respectively. Tazobactam was obtained from Wyeth (Pearl River, NY, USA). Sulbactam was a gift from Pfizer (Illertissen, Germany).

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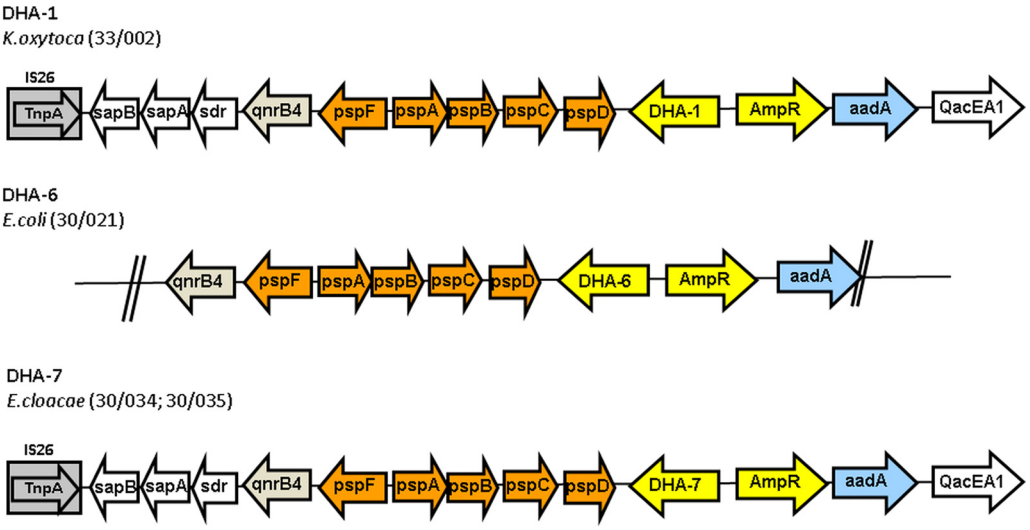

FIG 1 Representation of the genetic contexts of bla_{DHA-6} and bla_{DHA-7} and one of the bla_{DHA-1} structures detected in the same multicenter study.

Nitrocefin was obtained from Unipath Oxoid (Basingstoke, Hants, United Kingdom), and isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Roche (Basel, Switzerland).

Bacterial strains. *Klebsiella oxytoca* 33/002 was used for cloning the bla_{DHA-1} gene and for MIC analysis. It was isolated in the Hospital Universitari Joan XXIII de Tarragona, Tarragona, Spain. *Escherichia coli* 30/021 was used for cloning the bla_{DHA-6} gene and for MIC analysis. It was isolated in the Vall d'Hebron Hospital, Barcelona, Spain. *Enterobacter cloacae* 30/034 was used for cloning the bla_{DHA-7} gene and for MIC analysis. It was isolated in the Vall d'Hebron Hospital, Barcelona, Spain. *Escherichia coli* TG1 {*supE hsdΔ5 thi* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} ΔM15]} was used as the recipient in cloning experiments and for MIC analysis. *Escherichia coli* BL21 (*hsdS gal* [λ*cIts*857 *ind1 Sam7 nin5 lac* UV5-T7 gene 1]) was used for expression experiments.

***In vitro* susceptibility testing.** All the antibiotic MICs were determined by microdilution according to CLSI methodology (13) and confirmed by Etest (bioMérieux, Marcy l'Etoile, France), and the results were interpreted according to the manufacturer's recommendations. For inhibition studies, ampicillin was tested when indicated with fixed concentrations of clavulanic acid, sulbactam, and tazobactam (4 mg/liter).

Genetic contexts of the bla_{DHA} genes in *Enterobacteriaceae* isolates. Pulsed-field gel electrophoresis (PFGE) with S1 nuclease digestion of whole-genome DNA (S1-PFGE) and PCR-based replicon typing (PBRT) were used to characterize the plasmids, as previously described (14). PFGE with I-CeuI digestion of whole-genome DNA, as described by Liu et al. (15), was used to determine whether the bla_{DHA} genes were located in the chromosome.

The nucleotide sequences surrounding the bla_{DHA} genes were determined by PCR and were compared with previously described structures (12). Sequencing reactions were performed with the BigDye Terminator kit (PE Applied Biosystems, Foster City, CA), and sequences were analyzed in an ABI Prism 3100 DNA sequencer (PE Applied Biosystems). The resulting sequences were then compared with those available in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Cloning and DNA analysis. PCR techniques were used to obtain the bla_{DHA-1}, bla_{DHA-6}, and bla_{DHA-7} genes from *K. oxytoca* 33/002, *E. coli* 30/021, and *E. cloacae* 30/034, respectively. The genes were then cloned into plasmid pBGS18, which harbored an external promoter, pBGS18-Pctx (16). For cloning the DHA genes, we used the primers DHA-pBGS18 fw (5′-AAAAGGTACCATGAAAAAATCGTTATCTGCAACAC, forward)

TABLE 1 MICs of several antibiotics for DHA-1, DHA-6, and DHA-7 β-lactamases in different bacterial strains

Antibiotic	MIC (mg/liter) for[a]: *E. coli* TG1 pBGS18-pCTX	*K. oxytoca* 33/002 (DHA-1)	*E. coli* 30/021 (DHA-6)	*E. cloacae* 30/034 (DHA-7)	*E. coli* TG1 pBGS18-pCTX[b] (DHA-1)	*E. coli* TG1 pBGS18-pCTX (DHA-6)	*E. coli* TG1 pBGS18-pCTX (DHA-7)
Ampicillin	2	2,056	512	>2,056	1,028	256	2,056
Ampicillin-clavulanic acid[c]	2	2,056	512	>2,056	1,028	256	2,056
Ampicillin-sulbactam[c]	2	128	128	2,056	128	128	2,056
Ampicillin-tazobactam[c]	2	32	64	>2,056	64	128	2,056
Cephalothin	4	512	512	>1,028	1,028	256	1,028
Cefoxitin	1	128	32	64	32	128	64
Ceftazidime	0.06	4	16	128	32	4	128
Cefotaxime	0.06	4	4	32	8	1	32
Cefepime	<0.12	0.12	0.12	8	0.5	0.12	0.12
Aztreonam	0.12	16	2	128	8	2	32
Imipenem	<0.03	0.25	0.5	4	0.12	0.06	0.12

[a] Clinical isolates that harbor the β-lactamase gene are shown in parentheses.
[b] pBGS18, pCTX plasmid expressing the indicated β-lactamases under the control of an external promoter.
[c] Ampicillin was tested when indicated with fixed concentrations of clavulanic acid, sulbactam, or tazobactam (4 mg/liter).

TABLE 2 Kinetic data for the pure DHA-1, DHA-6, and DHA-7 β-lactamases

Antibiotic and kinetic parameter	Data (mean ± SD) for:		
	DHA-1	DHA-6	DHA-7
Nitrocefin			
K_m (μM)	84 ± 8	279 ± 17	88 ± 11
k_{cat} (s^{-1})	1,537 ± 120	2,319 ± 420	797 ± 67
k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	18.297	8.311	9.056
Ampicillin			
K_m (μM)	0.877 ± 0.342	0.925 ± 0.088	1.466 ± 0.397
k_{cat} (s^{-1})	1.346 ± 0.226	1.9 ± 0.26	1.475 ± 0.095
k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	1.534	2.05	1.006
Cephalothin			
K_m (μM)	39.21 ± 16.33	66.39 ± 23.23	35.586 ± 15.66
k_{cat} (s^{-1})	179 ± 23	198 ± 7	222 ± 2
k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	4.565	2.98	6.23
Cefotaxime			
K_m (μM)	0.0824 ± 0.005	0.153 ± 0.021	0.0927 ± 0.002
k_{cat} (s^{-1})	0.0907 ± 0.01	0.125 ± 0.02	0.177 ± 0.024
k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	1.1	0.816	1.9
Ceftazidime			
K_m (μM)	207.07 ± 54	106.74 ± 22.74	240 ± 72
k_{cat} (s^{-1})	1.748 ± 0.192	0.708 ± 0.115	2.003 ± 0.22
k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	0.0084	0.0063	0.0083
Cefoxitin			
K_m (μM)	0.265 ± 0.137	0.256 ± 0.066	0.25 ± 0.127
k_{cat} (s^{-1})	0.04349 ± 0.007	0.0486 ± 0.008	0.0412 ± 0.007
k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	0.163	0.189	0.1648
Clavulanic acid (IC_{50} [μM])	806 ± 394	361 ± 25	483 ± 279
Sulbactam (IC_{50} [μM])	3.17 ± 0.23	2.99 ± 0.32	3.97 ± 0.21
Tazobactam (IC_{50} [μM])	0.143 ± 0.007	0.124 ± 0.007	0.267 ± 0.044

and DHA-pBGS18 rv (5′-AAAAGAATTCTTATTCCAGTGCACTCAAA ATAGC, reverse), which introduced the restriction sites KpnI and EcoRI, respectively. For microbiological analysis, all constructions were transformed in *E. coli* TG1.

Cellular fraction isolation. Supernatant, periplasm, and cytoplasmic fractions were obtained from cultures of isogenic *E. coli* TG1 strains expressing the three DHA-type enzymes according to pET system manual instructions (Novagen, Darmstadt, Germany).

The QuantiChrom glucose-6-phosphate dehydrogenase kit from BioAssay Systems (Hayward, CA, USA) was used to rule out cellular contamination in the periplasmic and supernatant fractions.

Purification of DHA enzymes. To purify the DHA-1, DHA-6, and DHA-7 proteins, the three corresponding genes were cloned in the pGEX-6P-1 vector with the primers DHA-pGEX fw (5′-AAGAATTCGCTGAT AATGTCGCGG, forward) and DHA-pGEX rv (5′-AACTCGAGTTATT CCAGTGCACTC, reverse), which generated the restriction sites EcoRI and XhoI, respectively. The constructs were transformed in *E. coli* BL21 to produce a fusion between glutathione *S*-transferase (GST) and the DHA enzymes without their signal peptides. The β-lactamases were purified to homogeneity and the GST was removed from the DHA enzymes according to the manufacturer's instructions for the GST gene fusion system (Amersham Pharmacia Biotech Europe GmbH, Germany).

Determination of kinetic parameters. In order to monitor the hydrolysis of antibiotics by DHA β-lactamases, the variation in absorbance that resulted from the opening of the β-lactam ring was recorded under the following conditions. The antibiotic extinction coefficients for nitrocefin, ampicillin, cefotaxime, ceftazidime, cefoxitin, and cephalothin were 15,000, −820, −7,500, −9,000, −7,700, and −6,500 $M^{-1}cm^{-1}$, respectively, at wavelengths of 260 nm for cefotaxime, ceftazidime, cefoxitin, and cephalothin, 482 nm for nitrocefin, and 235 nm for ampicillin. The antibiotics were dissolved in phosphate-buffered saline (PBS) supplemented with 20 μg bovine serum albumin (BSA)/ml, and the tests were repeated three times at 25°C. The kinetic parameters for nitrocefin were determined by measuring the initial hydrolysis rates and using the Hanes-Woolf linearization of the Henri-Michaelis-Menten equation. For the other antibiotics, the K_m value was measured as the K_i in a competition experiment with nitrocefin as the reporter substrate. The k_{cat} values were obtained by monitoring the hydrolysis of the antibiotic at a concentration of ≫10 times the K_m. The 50% inhibitory concentration (IC_{50}) studies with clavulanic acid, sulbactam, and tazobactam were performed according to a previously described method and by using nitrocefin as the competitor substrate (17).

Stability experiments. Pure DHA-type enzymes were incubated at 50°C. The residual activity against nitrocefin was then measured at 10-min intervals for 40 min (16). Triplicate experiments were performed, and reported data are the mean values from three independent assays.

RESULTS AND DISCUSSION

During a national multicenter survey of AmpC β-lactamases, the bla_{DHA-1}, bla_{DHA-6}, and bla_{DHA-7} genes were located in three different strains of *Enterobacteriaceae*, *K. oxytoca*, *E. coli*, and *E. cloacae*, respectively (18). Plasmid characterization by PFGE (see Materials and Methods) revealed that the DHA-6 β-lactamase is encoded by a plasmid of 87.3 kb and the incompatibility group I1. The DHA-7 β-lactamase gene hybridized with a 310.4-kb plasmid of incompatibility group HI2. The genetic contexts of bla_{DHA-6} and bla_{DHA-7} were compared with those of 19 bla_{DHA-1}-producing isolates that were recovered during the same multicenter study (19). Although a certain degree of variability was detected, as reported in the relevant literature (10, 11, 12), the genetic structures that harbored the new bla_{DHA} genes were conserved relative to those of bla_{DHA-1} (Fig. 1).

bla_{DHA-6} and bla_{DHA-7} are the first novel DHA β-lactamases from Spain to be described in detail. However, this is not the first description of a DHA β-lactamase in this country. An outbreak of *K. pneumoniae* carrying DHA-1 was described as recently as 2013 (20). Nevertheless, the genetic context of bla_{DHA-1} was not reported in that study. The description of a bla_{DHA} gene in *E. coli* is not novel, as this gene was first described in Europe in 2006 (21).

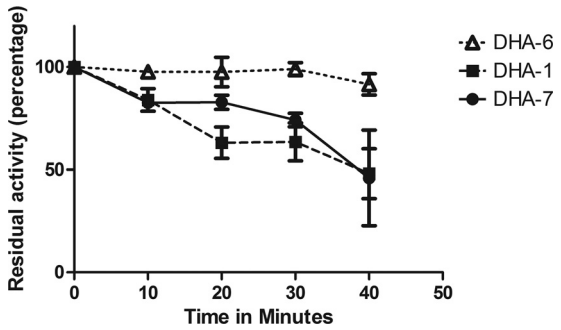

FIG 2 Percentage of residual activity of pure DHA-type enzymes after denaturation experiments at 50°C.

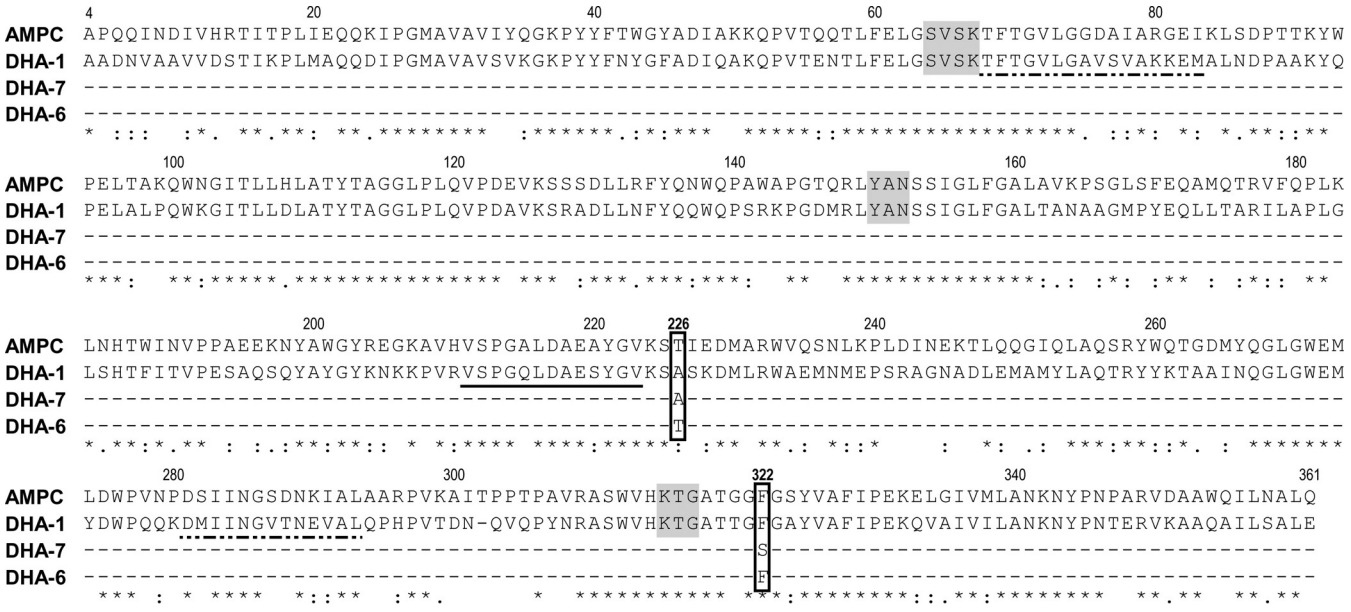

FIG 3 Amino acid sequence alignment of the DHA β-lactamase family (DHA-1, GenBank accession no. Y16410; DHA-6, HQ322612; DHA-7, HQ456945) with AmpC from *Escherichia coli*. Asterisks indicate strictly conserved amino acids, two dots indicate residues that are very similar, and one dot indicates residues that are more or less similar. Ala226Thr and Phe322Ser mutation positions are 0. The three classical conserved motifs are shaded in gray, the omega loop is indicated by a solid black line, and the H2 and H10 helices are represented by dashed lines. The alignment was created with the CLUSTAL W program of the EMBL-EBL.

However, our description of bla_{DHA-7} represents the first report of a bla_{DHA} gene in an *E. cloacae* strain. This discovery highlights the rapid spread of bla_{DHA} genes among *Enterobacteriaceae*. The mobilization factor for bla_{DHA} genes may be a transposable element. This conclusion is supported by the data showing that the genetic context is almost the same for the bla_{DHA} genes described in this paper.

For comparative microbiological analysis, the three bla_{DHA} genes were transformed into *E. coli* to yield an isogenic background. The MIC values of a large number of antibiotics were calculated for all the bacterial strains included in this study, including the original bacterial clinical isolates (Table 1).

Our results reveal slight differences between the isogenic bacterial isolates expressing DHA-type proteins, except for cefotaxime and ceftazidime. The DHA-1 and DHA-7 transformants were resistant to ceftazidime and cefotaxime, unlike DHA-6; the MICs were decreased 8- to 32-fold in DHA-6 relative to those in DHA-1 and DHA-7 (Table 1). There was also a 16-fold reduction in the MIC of aztreonam in DHA-6 relative to that in DHA-7. The differences were greatest between DHA-7 and DHA-6. Also, the cephalothin and ampicillin MICs were slightly lower for DHA-6. Interestingly, the cefoxitin MIC was higher for DHA-6 than for DHA-1 or DHA-7, and the cefepime MICs remained stable among the three different DHA-type enzymes. Microbiological analysis of the efficiencies of classical inhibitors showed that tazobactam and sulbactam exert some inhibitory activity against DHA-1 and DHA-6, as revealed by combination with ampicillin (Table 1).

The kinetic parameters of the purified DHA β-lactamases were determined for nitrocefin, ampicillin, cephalothin, cefotaxime, ceftazidime, and cefoxitin (Table 2). In general, the results revealed only small differences among the three proteins. The catalytic efficiency against nitrocefin is 2-fold higher in DHA-1 than in DHA-6 and DHA-7. The catalytic efficiencies for nitrocefin, ampicillin, cefotaxime, and especially cephalothin were good for the three enzymes, but there was a slight reduction in the efficiencies for cephalothin, cefotaxime, and ceftazidime of DHA-6 compared to those of DHA-1 and DHA-7. The IC_{50} constants showed slightly better inhibition for clavulanic acid and sulbactam in DHA-6 than in either DHA-1 or DHA-7 β-lactamase (Table 2). The stability of these DHA enzymes was assessed with temperature inactivation studies. These results show that the DHA-6 protein displayed a significantly higher stability than did DHA-1 or DHA-7. After 40 min of incubation at 50°C, DHA-6 retained 90% of residual activity on nitrocefin, compared to 48% and 45% for DHA-1 and DHA-7, respectively (Fig. 2).

As the kinetic data do not completely clarify the microbiological differences observed between DHA-6 and DHA-1 or DHA-7, the cellular fractions of the cultures of isogenic *E. coli* that expressed three DHA-type enzymes were obtained, and the specific activities against nitrocefin were calculated. The putative contamination of periplasmic components or supernatant with cytoplasmic components was ruled out by assaying glucose-6-phosphate dehydrogenase activity. The results revealed that periplasmic fractions of DHA-1 and DHA-7 were four to two times more specifically active against nitrocefin. Also, a clear remaining β-lactamase activity was observed in DHA-7 supernatants, which was contrary to absent activity in DHA-1 and DHA-6 supernatants. Accordingly, these data showed less DHA-6 enzyme in the periplasmic fractions than DHA-1 and DHA-7, which may indicate slight differences in the secretion of the different DHA-type enzymes. These differences may contribute, together with the different kinetic properties, to the higher MICs observed in DHA-1- and DHA-7-expressing *E. coli* strains than those in DHA-6-expressing *E. coli* strains in most of the β-lactams tested.

The three enzymes had mutations at only two positions. The

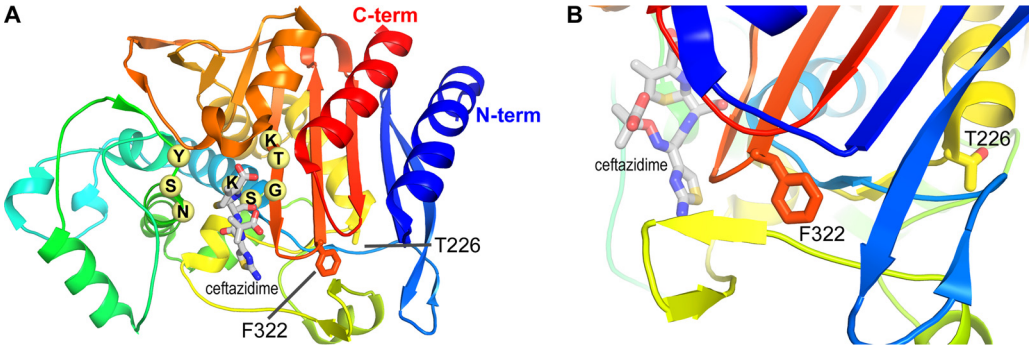

FIG 4 (A) AmpC structure in complex with ceftazidime represented as a cartoon with a rainbow coloring scheme (blue N terminus to red C terminus). Important residues that constitute the 3 conserved motifs appear as pale yellow spheres; ceftazidime appears as gray sticks. The 2 residues characteristic of the differences among DHA-1, DHA-6, and DHA-7 are also displayed as sticks. (B) Close-up view of the 2 residues of interest, with a 90° shift in orientation.

Ala226 present in DHA-1 and DHA-7 was replaced by a threonine in DHA-6, and the Phe322 found in DHA-1 and DHA-6 was mutated into a serine in DHA-7 (Fig. 3 and 4). AmpC from *E. coli* is the closest class C β-lactamase (61% amino acid identity) for which a three-dimensional structure is available. The two positions with modifications in the DHA studied (residues 226 and 322) are located in well-conserved areas and, respectively, corresponded to amino acids 226 and 322 in this structure (PDB code 2BLS) (22). Position 226 lies at the C-terminal end of an α helix in the α/β structure. In AmpC, this amino acid is a threonine, as in DHA-6. Position 322 lies 5 amino acids after the third conserved motif characteristic of the β-lactamases (KTG). It is a phenylalanine part of an internal hydrophobic cluster in AmpC that is also conserved in DHA-1 and DHA-6 (Fig. 3 and 4).There is no direct involvement of the mutated amino acid in substrate recognition or hydrolysis, and the stability studies showed that this effect is not caused by a loss of stability of the DHA-6 protein. In contrast, this protein is significantly more stable. According to the structural analysis of the AmpC structure, the Ala226Thr mutation characterizing DHA-6 is located at a junction with the N terminus of the polypeptide chain. The side chain of the threonine, therefore, may provide a better, complementary shape to stabilize the N-terminal part of the enzyme (Fig. 4, blue coloration) and better resist heat denaturation. This stabilization may induce a slight reduction of the dynamic of this enzyme and primarily affect the hydrolysis of some of the bulkiest antibiotics.

In summary, this work is the first detailed description of DHA-6 and DHA-7 β-lactamases and highlights the first isolation of a bla_{DHA} gene in *E. cloacae*. It is also the first detailed kinetic characterization of three highly similar DHA proteins. This study identified the new position 226 in DHA-type enzymes as relevant for the hydrolysis of certain cephalosporins, such as cephalothin, ceftazidime, and cefotaxime.

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