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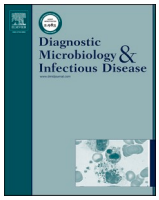

Original Article

Characterisation of a novel AmpC beta-lactamase, DHA-33, resistant to inhibition by cloxacillin

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ABSTRACT

Plasmid-encoded DHA-type AmpCs have been extensively reported in Enterobacterales. The expression of the genes encoding these plasmid-mediated enzymes are inducible and these enzymes are capable of conferring resistance to a wide spectrum of beta-lactams including penicillins and broad-spectrum cephalosporins. The identification of infections caused by AmpC-producing bacteria is a necessity, both for infection control/epidemiology purposes and to inform treatment choices. A common testing method for AmpC production in the clinical laboratory setting is to supplement Mueller-Hinton agar plates used for antibiotic disk diffusion with cloxacillin, a potent inhibitor of AmpC enzymes. Here we describe a novel DHA variant, produced by a clinical *Escherichia coli* isolate, which is resistant to cloxacillin inhibition.

1. Introduction

The first description of the Ambler class C DHA-type enzymes, namely DHA-1, was reported in 1997, in a *Salmonella enteritidis* isolate obtained from the stool sample of a patient in Saudi Arabia [1]. In this report, DHA-1 was found to be encoded on a conjugative plasmid and conferred resistance to a wide range of beta-lactams including the penicillins, amoxycillin and piperacillin, as well as the first (cefalothin), and third generation cephalosporins (ceftazidime and cefotaxime), and the cephamycin, cefoxitin [1]. The AmpC enzymes can be broadly split into two groups. There are those for which the expression of the corresponding genes are inducible with expression being regulated by a LysR-type *ampR* gene encoded in the opposite orientation (e.g. bla_{CMY}, bla_{DHA}). For others, the expression of the corresponding gene is not regulated by such system, hence are not inducible, but instead by promoter mutations (e.g. the *Escherichia coli* chromosomal *ampC*) [2–4]. Inducible AmpCs are found encoded within the chromosomes of a number of Gram-negative bacteria including *Enterobacter cloacae, Citrobacter freundii* and *Pseudomonas aeruginosa* amongst others. Whilst usually constitutively expressed at a low level, the expression of these genes are capable of being induced upon exposure to some beta-lactam antibiotics, facilitated by the *ampR* regulator gene [2–4]. DHA-type AmpCs are members of this inducible group and are derived from the chromosome of *Morganella morganii*, but since they have also been mobilised by mobile genetic structures, they are often reported on plasmids in Enterobacterales [2,3]. To date, 32 DHA variants have been identified, predominantly in *M. morganii*, but some also in Enterobacterale species, encoded on plasmids (http://bldb.eu/).

Cloxacillin is a potent inhibitor of AmpC enzymes and in the clinical laboratory setting, AmpC production is usually detected by exploiting this trait. Two common detection methods include antibiotic disk testing in the presence/absence of cloxacillin in the Mueller-Hinton agar plate, resulting in the inhibition of the AmpC and a significant increase in cephalosporin susceptibility, or using cefoxitin or cefotetan gradient MIC tests with and without a constant concentration of cloxacillin [5]. The identification of infections caused by AmpC-producing bacteria is a necessity, both for infection control/epidemiology purposes and to inform treatment choices.

In this study we describe a novel DHA variant, produced by a clinical *Escherichia coli* isolate, which is resistant to cloxacillin inhibition.

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2. Materials and methods

2.1. Bacterial isolates and antimicrobial susceptibility testing

A cephalosporin-resistant *E. coli* isolate, FR23, was sent to the Swiss National Reference Centre for Emerging Antibiotic Resistance (NARA) for further investigation. Species identification was confirmed using the EnteroPluri-Test (Liofilchem https://www.liofilchem.com) and UriSelect 4 agar (Bio-Rad, Cressier, Switzerland).

Susceptibility testing to beta-lactam antibiotics was performed by disc diffusion in the presence/absence of 250 mg/L cloxacillin, and MICs were determined by broth microdilution according to EUCAST guidelines [6].

2.2. Cloning experiments

The bla_{DHA} alleles, respectively encoding DHA-1 and DHA-33, and additionally the bla_{DHA} alleles and their respective *dhaR* gene, were amplified using primers DHA-Fw (5′- ACA CGG AAG GTT AAT TCT GA -3′) and DHA-Rev (5′-TTA TTC CAG TGC ACT CAA AAT AGC C -3′), DHA_AR_Fw (5′- TGA AGG TGA TGA TTT GCG GG -3′), DHA_AR_Rev (5′-GTC AGT GCC CGA TAC TCT CA -3′), and cloned into pCR-Blunt II-TOPO (Invitrogen, ThermoFisher), before transformation into *E. coli* Top10. Transformants were selected on plates supplemented with kanamycin (50 mg/L). Successful transformants were confirmed by PCR amplification and sequencing of the alleles.

2.3. Whole genome sequencing (WGS)

WGS was performed on a MiSeq (Illumina) instrument as previously described [7]. Assemblies were performed using the Shovill pipeline (https://github.com/tseemann/shovill) and contigs were annotated using Prokka [8]. STs, the presence of resistance genes and plasmid replicon types were determined using MLST 2.0, ResFinder 4.1 [9] and PlasmidFinder 2.1 [10], on the Center for Genomic Epidemiology platform (https://cge.cbs.dtu.dk/services/).

For long read sequencing, total genomic DNA (gDNA) of isolates was extracted from a bacterial culture grown overnight using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) and sequenced using the MinION Mk1B (Oxford Nanopore Technologies, Oxford, UK). Sequencing libraries were prepared a 1D chemistry Ligation Sequencing Kit (SQK-LSK109; Oxford Nanopore Technologies) and performed on a R9.4.1 Flongle Flow Cell (FLO-FLG001; Oxford Nanopore Technologies). Hybrid assembles, using both short and long-read data, were performed using UniCycler [11].

2.4. IC_{50} measurements

IC_{50} measurements using cloxacillin and tazobactam as ß-lactamase inhibitors were performed for DHA-1 and DHA-33. Briefly, β-lactamases from crude extracts were prepared and used for the measurement of the specific activity in 100 mM sodium phosphate (pH 7.0). Measurements were performed in a Genesys 10S UV/VIS spectrophotometer (Thermo Scientific) spectrophotometer using a wavelength of 262 nm for cephalothin. The 50 % inhibitory concentration (IC_{50}) for DHA variants was determined as the concentration of cloxacillin that reduced the hydrolysis rate of 100 μM cephalothin (CEF) by 50 %. Extracts were pre-incubated with inhibitor for 3 min prior to the addition of CEF and all measurements were performed in triplicate.

3. Results and discussion

3.1. Phenotypic and genotypic profiling of FR23

Isolate FR23 was an *E. coli* obtained from a rectal screening swab from a 39-year-old male in Switzerland. Susceptibility testing, performed by disk diffusion, showed that the isolate was resistant to all tested penicillins, including the penicillin-inhibitor combinations (eg. amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, piperacillin-tazobactam), cephalosporins, and aztreonam, but remained sensitive to the carbapenems. Additionally, the isolate was resistant to cefiderocol, exhibiting an MIC of 8 mg/L. No inhibition was observed in the presence of 250 mg/L cloxacillin supplemented in the Mueller-Hinton agar plate – usually used as an indicator of AmpC production, and so AmpC involvement was initially ruled out. PCRs targeting CTX-M encoding genes remained negative therefore the isolate was subject to whole genome sequencing (WGS) to allow for further detailed characterisation. WGS identified this isolate as belonging to ST9748, a single locus variant of ST131, and harboured a novel DHA variant, subsequently designated DHA-33 (GenBank Accession No. OR715113). The *E. coli* ST131 clonal complex is a multidrug resistant globally dominant clonal group, responsible for millions of infections annually, and is frequently reported to be increasing in prevalence [12]. DHA-33 differed from DHA-1 by a single amino acid at position E239K (or E219K according to the standard class C beta-lactamase numbering scheme [13]), located within the omega loop. The omega loop in class A and class C beta-lactamases is located in close proximity to the enzyme active site and is known to play a fundamental role in substrate specificity [14,15]. To date, of the thirty-three DHA variants (including DHA-33) identified, just four others harboured substitutions within the omega loop, three from *M. morgannii* (DHA-16, -19, and -30) and one identified in a *Citrobacter freundii* isolate (DHA-29), although none of these have been characterised phenotypically. Plasmid analyses identified a number of replicons present (I1, FIA, FIB(AP001918), FII(pRSB107), and Col156) in the FR23 clinical isolate. Combined short and long read sequencing allowed the closure of the DHA-33 encoding plasmid, subsequently named pDHA-33. This plasmid is a 160,347-bp multi-replicon plasmid (FIA, FIB(AP001918), FII(pRSB107), Col156) that also harboured the *qnrB4, sul1, mphA, tetB, catA1*, and *dfrA17* resistance genes. The bla_{DHA-33} gene was embedded within a class 1 integron and flanked by IS*26* and IS*6100* elements, similarly to what has been previously observed in the genetic environment of DHA-1 encoding plasmids [2]. pDHA-33 encodes a ~34 kb *tra* region and was predicted to be conjugative by MOB-Typer [16]. Additionally, pDHA-33 also encoded four type II toxin-anti-toxin systems (*ccdAB, pemIK*, and two copies of *vapBC*), likely contributing to plasmid stability [17]. A disruption in the chromosomally-encoded siderophore receptor *cirA* was also identified which is likely to contribute to the cefiderocol resistance observed in FR23. Mutations in the siderophores receptors such as *cirA* and *fiu* in *E. coli* have previously been associated with decreased susceptibility to cefiderocol [18]. The genotypic characteristics of both the clinical isolate and pDHA-33 are summarised in Table 1.

Table 1
Genotypic characteristics of FR23 and pDHA-33.

Strain/ Plasmid	ST	Size (bp)	Resistance genes	Replicon types
FR23	9748	NA	bla_{DHA-33}, *qnrB4, sul1, dfrA17, tetB, mphA, catA1*	I1, FIA, FIB(AP001918), FII(pRSB107), Col156
pDHA-33	NA	160, 347	bla_{DHA-33}, *qnrB4, sul1, dfrA17, tetB, mphA, catA1*	FIA, FIB(AP001918), FII (pRSB107), Col156

NA; not applicable.

3.2. Phenotypes of recombinant E. coli strains producing DHA-1 and DHA-33, pDHA-33, and relative enzyme kinetic measurements

Both bla_{DHA-1} and bla_{DHA-33} genes were cloned with and without their respective *ampR* (*dhaR*) regulatory gene, and further expressed in *E. coli* strains. When considering the recombinant strains producing the DHA variants alone, susceptibility testing showed that both variants

Table 2
MICs of the clinical isolate, recombinant strains expressing bla_{DHA-1} and bla_{DHA-33}, and the transconjugant.

	MIC (mg/L)																
	PIP	PTZ	TIC	TCC	AMX	AMC	TEM	CEF	FOX	CTX	CAZ	CZA	FEP	ATM	ATMA	ETP	FDC	FDCA
FR23	>256	256	>256	>256	>256	>256	>256	>256	256	128	>256	4	0.5	256	2	0.125	8	0.5
Top10	2	2	4	4	4	4	8	4	2	≤0.06	0.125	0.125	≤0.06	0.125	0.125	≤0.06	≤0.06	≤0.06
Top10/pTOPO-DHA-1	64	2	64	64	>256	>256	8	>256	4	4	8	1	≤0.06	0.5	0.125	≤0.06	≤0.06	≤0.06
Top10/pTOPO-DHA-33	16	2	>256	>256	256	256	32	128	4	4	8	1	≤0.06	2	0.125	≤0.06	0.125	≤0.06
Top10/pTOPO-DHA-1-DHA-R	128	16	>256	>256	>256	>256	16	>256	32	32	32	2	0.125	4	0.25	≤0.06	0.125	≤0.06
Top10/pTOPO-DHA-33-DHA-R	64	4	>256	>256	>256	>256	128	>256	64	32	32	2	0.125	16	0.25	≤0.06	0.5	≤0.06

PIP, piperacillin; PTZ, piperacillin-tazobactam; TIC, ticarcillin; TCC, ticarcillin-clavulanic acid; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; TEM, temocillin; CEF, cephalothin; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; CZA, ceftazidime-avibactam; FEP, cefepime; ATM, aztreonam; ATMA, aztreonam-avibactam; ETP, ertapenem; FDC, cefiderocol; FDCA, cefiderocol-avibactam.

Table 3
Inhibitory concentration of cloxacillin against DHA-1 and DHA-33.

Enzyme	Cloxacillin IC_{50} (μM)
DHA-1	0.002
DHA-33	8.887

conferred similar phenotypes. However, differences were observed for amoxicillin, piperacillin, and cephalothin, to which DHA-33 exhibited less activity relative to DHA-1 (Table 2). Conversely, the DHA-33-producing recombinant *E. coli* strain exhibited significantly higher MICs to ticarcillin, temocillin and aztreonam. In the recombinant strains containing both the bla_{DHA} genes and their respective regulatory genes, an overall increase in MICs was observed for all beta-lactams, including the differences between DHA-1 and DHA-33. Notably, there were significant differences between MICs to piperacillin-tazobactam (16 mg/L v 4 mg/L), aztreonam (4 mg/L v 16 mg/L), and cefiderocol (0.125 mg/L v 0.5 mg/L). The increased activity of DHA-33 against cefiderocol, combined with the disruption in *cirA*, could explain the resistant MIC of 8 mg/L observed in isolate FR23.

Enzymatic assays showed that the inhibitory activity of cloxacillin was considerably decreased against DHA-33 compared to DHA-1 (Table 3), with a >4000-fold increase in IC_{50}, explaining the lack of inhibition observed on the disk diffusion plates. Similar assays performed with tazobactam showed no difference between DHA-1 and DHA-33, and so it was determined that the decreased piperacillin-tazobactam MIC observed with the DHA-33 strain was not due to a reduction in the activity of tazobactam.

4. Conclusions

In this study we described a novel DHA variant, namely DHA-33, that exhibits increased activity against aztreonam and cefiderocol relative to DHA-1. Nevertheless, the aztreonam/avibactam combination still showed excellent effectiveness against such DHA-like producers, as well as the novel combinations containing a carbapenem and a newly-developed ß-lactamase inhibitor, such as meropenem/vaborbactam and imipenem/relebactam (data not shown). DHA-33 was resistant to inhibition by cloxacillin allowing the enzyme to initially evade identification by routine methods for AmpC activity. The dissemination of such inducible cloxacillin-resistant AmpC variants is concerning and could be problematic for both epidemiological surveillance and infection management.

Transparency of declarations

None to declare.

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CRediT authorship contribution statement

Jacqueline Findlay: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Laurent Poirel:** Writing – review & editing, Funding acquisition, Formal analysis, Conceptualization. **Abdessalam Cherkaoui:** Writing – review & editing, Formal analysis, Conceptualization. **Jacques Schrenzel:** Writing – original draft, Formal analysis, Conceptualization. **Patrice Nordmann:** Writing – review & editing, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

All authors: none to declare.

Data availability

The sequence read files and the sequence of plasmid pDHA-33 have been deposited to Genbank (BioProject no. PRJNA1111799 and PP795980).

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