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MECHANISMS OF RESISTANCE

Dissemination and Characteristics of a Novel Plasmid-Encoded Carbapenem-Hydrolyzing Class D β-Lactamase, OXA-436, Found in Isolates from Four Patients at Six Different Hospitals in Denmark

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ABSTRACT The diversity of OXA-48-like carbapenemases is continually expanding. In this study, we describe the dissemination and characteristics of a novel carbapenem-hydrolyzing class D β-lactamase (CHDL) named OXA-436. In total, six OXA-436-producing *Enterobacteriaceae* isolates, including *Enterobacter asburiae* ($n = 3$), *Citrobacter freundii* ($n = 2$), and *Klebsiella pneumoniae* ($n = 1$), were identified in four patients in the period between September 2013 and April 2015. All three species of OXA-436-producing *Enterobacteriaceae* were found in one patient. The amino acid sequence of OXA-436 showed 90.4 to 92.8% identity to the amino acid sequences of other acquired OXA-48-like variants. Expression of OXA-436 in *Escherichia coli* and kinetic analysis of purified OXA-436 revealed an activity profile similar to that of OXA-48 and OXA-181, with activity against penicillins, including temocillin; limited or no activity against extended-spectrum cephalosporins; and activity against carbapenems. The $bla_{OXA-436}$ gene was located on a conjugative ~314-kb IncHI2/IncHI2A plasmid belonging to plasmid multilocus sequence typing sequence type 1 in a region surrounded by chromosomal genes previously identified to be adjacent to bla_{OXA} genes in *Shewanella* spp. In conclusion, OXA-436 is a novel CHDL with functional properties similar to those of OXA-48-like CHDLs. The described geographical spread among different *Enterobacteriaceae* and the plasmid location of $bla_{OXA-436}$ illustrate its potential for further dissemination.

KEYWORDS carbapenems

Ambler class D β-lactamases constitute a large and diverse group of β-lactamases frequently identified among clinically relevant Gram-negative bacteria either as intrinsic chromosomally encoded β-lactamases in certain species or as acquired β-lactamases associated with mobile genetic elements (1, 2). In *Enterobacteriaceae*, the

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acquired carbapenem-hydrolyzing class D β-lactamases (CHDLs) have disseminated widely since the first identification of OXA-48 in a *Klebsiella pneumoniae* isolate (3) and have become the predominant carbapenemase type in many regions and countries (4). To date, several closely related OXA-48-like variants (e.g., OXA-48, OXA-162, OXA-163, OXA-181, OXA-204, OXA-232, OXA-244, OXA-245, OXA-247, OXA-370, and OXA-405) have been identified in clinically relevant *Enterobacteriaceae* (1, 5, 6). The bla_{OXA-48}-like genes have been associated with different transposons formed by insertion sequences (IS), such as IS*1999* and IS*Ecp1*, and for bla_{OXA-48}, an association with IncL plasmids has been described (1, 7, 8). Several studies have identified *Shewanella* spp., environmental waterborne bacteria, to be the natural host of bla_{OXA-48}-like genes (9–11).

The amino acid sequence similarities between acquired OXA-48-like variants are high (>97%). In general, the hydrolytic activities of OXA-48-like enzymes are similar to those of other CHDLs, with activities against penicillins and carbapenems but no or limited activity against extended-spectrum cephalosporins (2, 12). The activity against carbapenems is variable but, in general, low, which, in the absence of additional mechanisms (i.e., membrane permeability defects), often results in a carbapenem-susceptible phenotype (12). Three variants, OXA-163, OXA-247, and OXA-405, deviate from this general profile, as they have clavulanic acid-inhibited activity against extended-spectrum cephalosporins and even lower or negligible activity against carbapenems (6, 13, 14).

In this study, we describe a novel plasmid-mediated OXA-48 variant named OXA-436 initially discovered in an *Enterobacter asburiae* isolate recovered from a patient admitted to a hospital in the Capital Region of Denmark and subsequently detected in isolates from three other patients in Denmark, including other *Enterobacteriaceae*.

RESULTS AND DISCUSSION

Epidemiological setting and identification of $bla_{OXA-436}$. In September 2013, an *E. asburiae* isolate (AMA 497) intermediate susceptible to meropenem was detected from a urine sample from a patient (patient 1) (Table 1). In January 2014, a second *E. asburiae* isolate (AMA 590), also intermediate susceptible to meropenem, was detected from another patient (patient 2) at another hospital. Both isolates were positive for carbapenemase production by the Carba NP test, and the phenotypic data indicated the presence of a class D carbapenemase (see below). However, the isolates were negative for bla_{OXA-48} by using previously described internal primers (3), as well as negative for genes encoding other known carbapenemases, bla_{VIM}, bla_{IMP}, bla_{GIM}, bla_{SPM}, bla_{SIM}, bla_{NDM}, and bla_{KPC}, by PCR. In order to identify the carbapenemase, the isolates were subjected to whole-genome sequencing (WGS), and the sequence data (15) were analyzed using the ResFinder program (16), which indicated the presence of a gene for a β-lactamase with 88% nucleotide sequence similarity to $bla_{OXA-181}$ (15). Subsequent analysis of the nucleotide sequence and comparison of its nucleotide sequence with the nucleotide sequences of the genes for other OXA-48-like variants showed that it encoded a class D β-lactamase with close similarity to other OXA-48-like variants and was designated OXA-436 (Fig. 1). A new PCR for the detection of $bla_{OXA-436}$ was subsequently developed and integrated into the PCR analysis of isolates suspected of harboring a carbapenemase submitted to the Danish National Reference Laboratory. Subsequently, four isolates recovered from two other patients in the period between August 2014 and April 2015 were PCR positive for $bla_{OXA-436}$ (Table 1).

In August 2014, a $bla_{OXA-436}$ PCR-positive *Citrobacter freundii* isolate was detected from a third patient (patient 3). During October and November 2014, a $bla_{OXA-436}$-positive *K. pneumoniae* isolate and a $bla_{OXA-436}$ positive *E. asburiae* isolate were detected from patient 3. In April 2015, a $bla_{OXA-436}$-positive *C. freundii* isolate (AMA 948) was recovered from a fourth patient (patient 4). The three *E. asburiae* isolates had highly similar single nucleotide polymorphism (SNP) profiles (4 to 15 SNP differences), indicating clonal spread. In contrast, the two *C. freundii* isolates belonged to two different sequence types (STs; ST22 and ST65) and had very different SNP profiles (>29,000 SNP differences) (Table 1), indicating independent events of plasmid transfer.

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TABLE 1 Description of the six $bla_{OXA-436}$-positive isolates from four patients

Patient no.	Species	Reference designation	Sample collection date	Hospital	Specimen	MLST ST	SNP profile	β- Lactamases	Other resistance genes
1	*E. asburiae*	AMA 497	Sept. 2013	A	Urine			$bla_{ACT-6-like}$, bla_{OXA-10}, bla_{SHV-12}, bla_{TEM-1b}	*strA, strB, aac(6′)-Ilc, aadA1, aadA2, qnrA1, sul1, sul2, dfrA18*
2	*E. asburiae*	AMA 590	Jan. 2014	B	Urine		4 SNP differences from AMA 497	$bla_{ACT-6-like}$, bla_{OXA-10}, bla_{SHV-12}, bla_{TEM-1b}	*strA, strB, aac(6′)-Ilc, aadA1, aadA2, qnrA1, sul1, sul2, dfrA18*
3	*C. freundii*	AMA 754	Aug. 2014	C	Pleural fluid	ST65		$bla_{CMY-48-like}$, bla_{OXA-10}, bla_{SHV-12}, bla_{TEM-1b}	*strA, strB, aac(6′)-Ilc, aadA1, aadA2, qnrA1, sul1, sul2, dfrA18*
3	*K. pneumoniae*	AMA 782	Oct. 2014	D	Pleural fluid	ST35		bla_{SHV-1}, bla_{OXA-10}, bla_{SHV-12}, bla_{TEM-1b}	*strA, strB, aac(6′)-Ilc, aadA1, aadA2, qnrA1, sul1, sul2, dfrA18*
3	*E. asburiae*	AMA 810	Nov. 2014	E	Expectorate		15 SNP differences from AMA 497	$bla_{ACT-6-like}$, bla_{OXA-10}, bla_{SHV-12}, bla_{TEM-1b}	*strA, strB, aac(6′)-Ilc, aadA1, aadA2, qnrA1, sul1, sul2, dfrA18*
4	*C. freundii*	AMA 948	Apr. 2015	F	Urine	ST22	>29,000 SNP differences from AMA 754	$bla_{CMY-48-like}$, bla_{OXA-10}, bla_{SHV-12}, bla_{TEM-1b}	*strA, strB, aac(6′)-Ilc, aadA1, aadA2, qnrA1, sul1, sul2, dfrA1, dfrA18*

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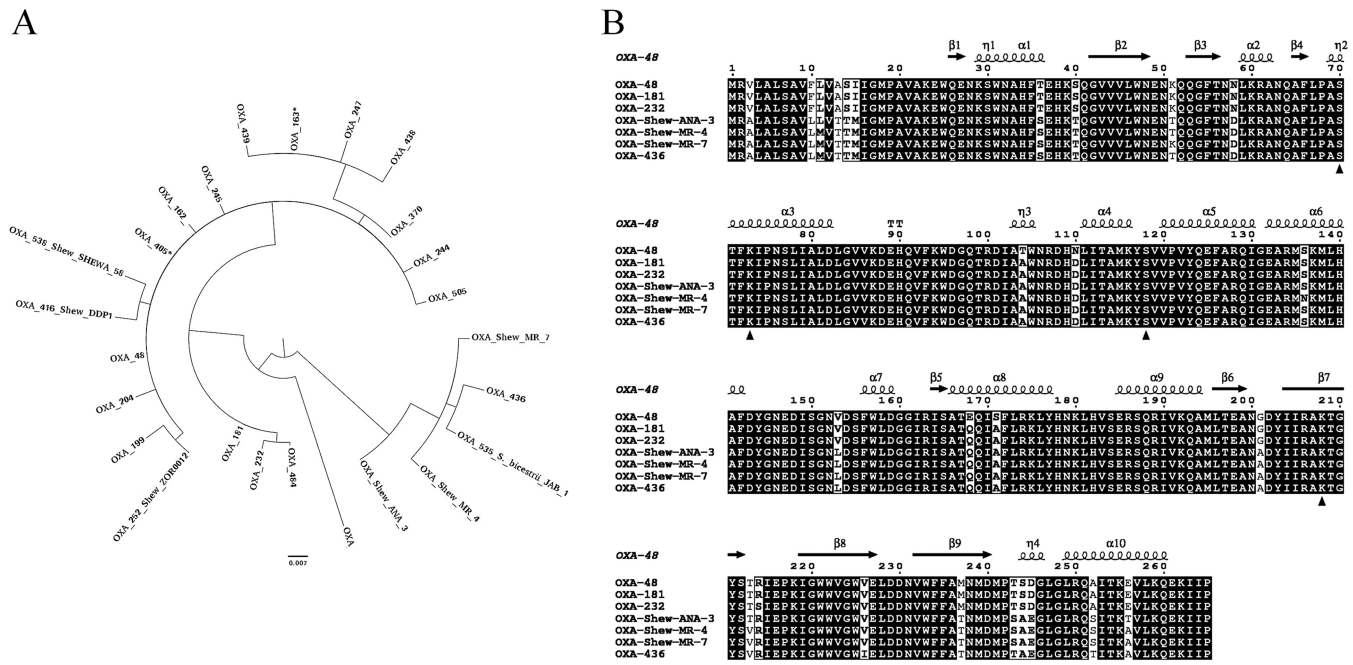

FIG 1 (A) Phylogenetic tree based on amino acid sequences showing the relationship of OXA-436, acquired OXA-48-like variants, and selected OXA-48-like variants from *Shewanella* spp. The tree is rooted at the midpoint. OXA-48-like variants devoid of carbapenemase activity are indicated with asterisks. (B) Alignment of the amino acid sequence of OXA-436 with the amino acid sequences of selected OXA-48-like variants. Conserved motifs are shaded in black. The secondary structural elements of OXA-48 are indicated above the sequences.

All six OXA-436-producing isolates were from clinical specimens and were from six different hospitals (Table 1). Unfortunately, none of the hospitals screened the patients by collection of a rectal swab sample for outbreak investigation. A retrospective analysis of selected isolates submitted prior to the first identification of $bla_{\text{OXA-436}}$ was performed but revealed no $bla_{\text{OXA-436}}$-positive isolates. None of the four patients with OXA-436-producing isolates had recently traveled abroad, and the origin of the $bla_{\text{OXA-436}}$ gene remains unknown.

Phenotypic profiles and molecular characteristics of $bla_{\text{OXA-436}}$-positive isolates. The antimicrobial susceptibility profiles of the $bla_{\text{OXA-436}}$-positive isolates are summarized in Table 2. All isolates were resistant to penicillins (including high-level resistance to temocillin), extended-spectrum cephalosporins, and aztreonam. Furthermore, all isolates were resistant to ertapenem but were either susceptible or intermediately susceptible to meropenem and imipenem. Nonsusceptibility to gentamicin, tobramycin, ciprofloxacin, and trimethoprim-sulfamethoxazole was also observed. All isolates retained susceptibility to ceftazidime-avibactam, colistin, amikacin, and tigecycline, except for the *C. freundii* isolate, which was intermediately susceptible to tigecycline. The non-β-lactam β-lactamase inhibitor avibactam has been shown to be a potent inhibitor of class A, class C, and certain class D enzymes (17). To assess the inhibitory properties of avibactam on OXA-436, determination of the MICs of temocillin and meropenem with and without avibactam were performed since OXA-48-like enzymes have limited or no activity against ceftazidime (12) and since the strains carried multiple other β-lactamases. For the clinical isolates, the additive inhibitory effect of 4 mg/liter avibactam reduced the temocillin MIC 16- to 32-fold and the meropenem MIC 16- to 64-fold. For the cloned OXA-436-producing *Escherichia coli* TOP10 isolate, the temocillin MIC was reduced 16-fold; however, the lower endpoint of the test range for meropenem allowed only a $\geq$2-fold MIC reduction to be observed. The results of phenotypic synergy tests for β-lactamase-mediated resistance were either inconclusive or negative, with the exception of positive test results for extended-spectrum β-lactamases (ESBLs) (data not shown). No synergy between meropenem and boronic acid, dipicolinic acid, or cloxacillin was observed. In addition, no zone diameter was

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TABLE 2 Antimicrobial susceptibility profiles of six clinical isolates, an *E. coli* J53A transconjugant of AMA 754 (AMA 1292), and *E. coli* J53A and β-lactam susceptibilities of *E. coli* TOP10 expressing β-lactamases and *E. coli* TOP10

Antibiotic	MIC (mg/liter)											
	E. asburiae AMA 497	*E. asburiae* AMA 590	*C. freundii* AMA 754	*K. pneumoniae* AMA 782	*E. asburiae* AMA 810	*C. freundii* AMA 948	*E. coli* AMA 1292[a]	*E. coli* J53A3R	*E. coli* TOP10 (pCR-BluntII-OXA-436)	*E. coli* TOP10 (pCR-BluntII-OXA-48)	*E. coli* TOP10 (pCR-BluntII-OXA-181)	*E. coli* TOP10
Temocillin	>256	>256	>256	>256	>256	>256	>256	8	>256	>256	>256	16
Piperacillin-tazobactam	>64	>64	>64	>64	>64	>64	>64	2	>64	>64	>64	2
Ceftazidime	>32	>32	32	>32	>32	>32	>32	0.25	0.5	1	1	0.5
Ceftazidime-avibactam	0.5	0.25	0.5	0.5	0.25	1	0.5	0.125	0.25	0.5	1	0.25
Cefotaxime	8	8	8	4	8	>16	4	≤0.06	0.125	1	2	0.125
Cefoxitin	>32	>32	>32	16	>32	>32	16	8	16	16	32	4
Aztreonam	>16	>16	>16	>16	>16	>16	>16	≤0.125	≤0.125	0.25	0.25	≤0.125
Meropenem	4	4	1	2	4	1	1	0.032	0.5	1	1	0.032
Imipenem	4	4	2	2	4	2	2	0.25	2	2	2	0.25
Ertapenem	>4	4	4	>4	>4	4	4	≤0.015	1	2	2	≤0.015
Gentamicin	128	128	64	128	64	64	32	≤0.25	ND[b]	ND	ND	ND
Amikacin	<1	<1	<1	<1	<1	<1	<1	<1	ND	ND	ND	ND
Tobramycin	4	8	8	8	8	4	2	<0.5	ND	ND	ND	ND
Ciprofloxacin	4	4	8	16	4	2	0.5	<0.015	ND	ND	ND	ND
Tigecycline	0.5	0.5	2	1	1	0.5	0.25	0.25	ND	ND	ND	ND
Colistin	0.5	0.5	1	0.5	0.5	1	≤0.25	0.5	ND	ND	ND	ND
Trimethoprim-sulfamethoxazole	>16	>16	>16	>16	>16	>16	>16	>16	ND	ND	ND	ND

[a]AMA 1292 is the *E. coli* J53A transconjugant of AMA 754.

[b]ND, not determined.

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observed with temocillin, indicating the presence of an OXA-48-like carbapenemase (18). All isolates were Carba NP test positive, illustrating the usefulness of this method for the detection of novel carbapenemases. Although some studies have reported problems with the low sensitivity of this test for OXA enzymes (19, 20), the enzyme activity of OXA-436 was sufficient for a positive Carba NP test result, and positive reactions were observed in <30 min. Similarly, positive test results were also obtained for all six study isolates by a novel immunochromatographic test (OXA-48 *K*-SeT; Coris BioConcept) for the detection of OXA-48-like enzymes. This showed that the monoclonal anti-OXA-48 antibodies incorporated in this lateral flow-based test also bind to the OXA-436-containing conjugate.

Genetic context of *bla*$_{\text{OXA-436}}$. The identification of *bla*$_{\text{OXA-436}}$ in different species, including the identification of three different *bla*$_{\text{OXA-436}}$-positive species from the same patient (patient 3), indicated that *bla*$_{\text{OXA-436}}$ is plasmid borne and that transfer can occur *in vivo*. Further, the identification of a common set of acquired resistance genes (Table 1) among the isolates indicated colocalization on the same mobile genetic element. Replicon identification using the PlasmidFinder web tool also showed the presence of IncHI2 and IncHI2A replicons in all strains. *In vitro* plasmid transfer experiments supported the plasmid localization of *bla*$_{\text{OXA-436}}$, as transconjugants were obtained from three donor strains (*C. freundii* AMA 754, *K. pneumoniae* AMA 782, and *E. asburiae* AMA 810). This was confirmed by combined single-molecule real-time (SMRT; PacBio) and MiSeq (Illumina) sequencing to generate a circular plasmid (designated pOXA436) genome of 314,137 bp (Fig. 2A) harboring *bla*$_{\text{OXA-436}}$. pOXA436 were found to be an IncHI2 plasmid belonging to ST1 according to the plasmid double-locus sequence typing scheme for IncHI2 plasmids (21). Comparison of pOXA436 with other IncHI2 plasmids showed the presence of the common backbone found in other IncHI2 plasmids (see, e.g., references 21, 22, 23, 24), including the replicon IncHI2, transfer regions Tra1 and Tra2, a partitioning system, and regions conferring resistance to heavy metals (tellurite, mercury, and arsenic). IncHI2 plasmids have previously been associated with a large number of various antibiotic resistance genes, including genes for carbapenemases, such as *bla*$_{\text{VIM}}$, *bla*$_{\text{IMP}}$, and *bla*$_{\text{NDM}}$ (21, 25). In pOXA436, the antibiotic resistance genes were located in two separate regions of the plasmid (Fig. 2A). In contrast to the neighboring genetic structures of *bla*$_{\text{OXA-48}}$-like genes identified in *Enterobacteriaceae*, which are closely associated with IS elements, such as IS*1999* and IS*Ecp1* (1), the *bla*$_{\text{OXA-436}}$ gene was located in a region of >7,300 bp surrounded by chromosomal genes previously identified to be adjacent to *bla*$_{\text{OXA}}$ genes in *Shewanella* (9). This region included an open reading frame encoding a hypothetical protein and the *sprT* gene, encoding a protein of unknown function, upstream of *bla*$_{\text{OXA-436}}$. Downstream of *bla*$_{\text{OXA-436}}$, a gene encoding a putative LysR-type transcriptional regulator was followed by a truncated *acc* gene encoding a carbamoyl phosphate synthase. Overall, this region showed high nucleotide sequence similarity (96%) with the chromosomal region in *Shewanella* sp. strains MR-4 and MR-7, isolated from the Black Sea; *Shewanella* sp. strain ANA-3, isolated from arsenic-treated wood in a brackish estuary in the United States (26); and a clinical *Shewanella bicestrii* sp. nov. isolate, JAB-1 (GenBank accession number CP022358). The species of strains MR-4, MR-7, and ANA-3 have not been determined, but genome analysis indicates that these could represent new *Shewanella* species (26). Interestingly, a novel OXA-48-like enzyme, OXA-535, with 98.9% amino acid sequence identity to OXA-436 was identified in *Shewanella bicestrii* sp. nov. strain JAB-1 (27), suggesting that this might be the original host and that *bla*$_{\text{OXA-535}}$ is the progenitor of *bla*$_{\text{OXA-436}}$. The exact mechanism behind the mobilization of the *bla*$_{\text{OXA-436}}$ region onto the pOXA436 plasmid is unclear. However, two IS*91* family-like IS elements (ISCR1) were identified upstream of the *bla*$_{\text{OXA-463}}$ region and one was identified downstream of the *bla*$_{\text{OXA-463}}$ region, and these elements could have been involved in the mobilization of the *bla*$_{\text{OXA-436}}$ region onto the pOXA436 plasmid (Fig. 2B). The transposase genes of all three IS*91*-like/ISCR1 elements were

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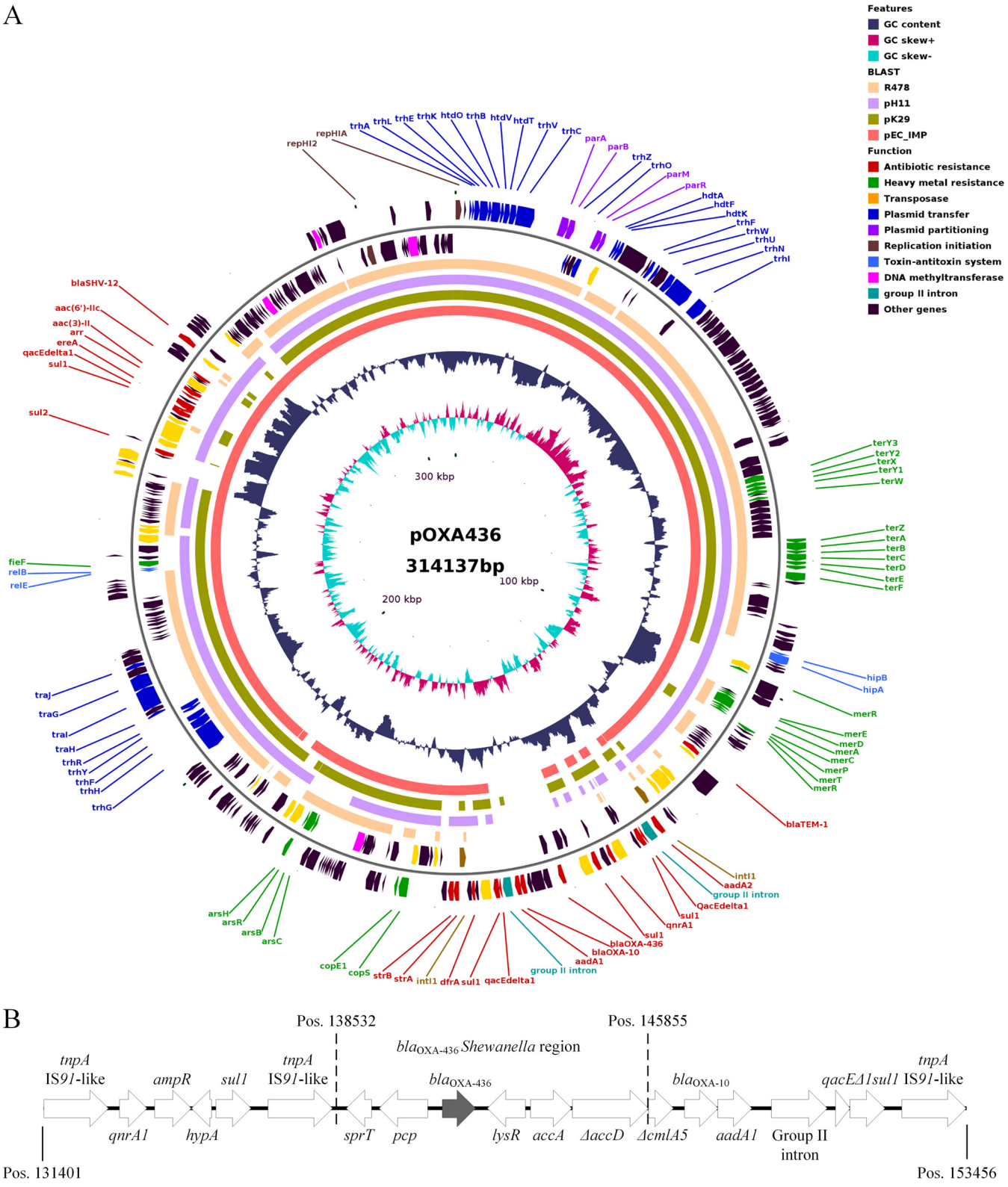

FIG 2 (A) Circular map of pOXA436 (GenBank accession number KY863418) drawn using CGView (47). The two outer circles show predicted open reading frames, with selected genes indicated according to gene function (see the key for the color scheme). The next four circles show homology to other IncHI2 plasmids: R478 (GenBank accession number BX664015), pH11 (GenBank accession number CP013215), pK29 (GenBank accession number EF382672), and pEC_IMP (GenBank accession number EU855787). (B) Linear representation of the genetic structure surrounding the $bla_{OXA-436}$ region in pOXA436. Arrows represent open reading frames and the direction of transcription. Horizontal dotted lines mark the boundaries of the region with a high level of identity to *Shewanella* isolates MR-4 (GenBank accession number CP000446), MR-7 (GenBank accession number CP000444), and ANA-3 (GenBank accession number CP000469) and *S. bicestrii* sp. nov. JAB-1 (GenBank accession number CP022358). The nucleotide positions of the fragment in relation to the pOXA436 sequence are indicated.

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TABLE 3 Kinetic parameters of OXA-436 compared to OXA-48 and OXA-181

Subtrate	K_m (μM)			k_{cat} (s^{-1})			k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)		
	OXA-436	OXA-48[a]	OXA-181[b]	OXA-436	OXA-48[a]	OXA-181[b]	OXA-436	OXA-48[a]	OXA-181[b]
Benzylpenicillin	200 ± 100	79	90	900 ± 100	446	444	4.5	5.6	5.0
Ampicillin	5 ± 1	395	170	600 ± 40	955	218	120	2.4	1.3
Temocillin	200 ± 100	45	60	3 ± 1	0.3	0.3	0.015	0.0066	0.005
Cefotaxime	130 ± 20	190	>1000	6 ± 0.4	10	>62	0.046	0.06	0.013
Ceftazidime	150 ± 50	5100	NH[c]	7 ± 1	4	ND[d]	0.047	0.001	ND
Imipenem	20 ± 1	13	13	6 ± 0.2	4.8	7.5	0.3	0.37	0.55
Meropenem	3 ± 1	11	70	0.14 ± 0.01	0.07	0.1	0.047	0.0062	0.0015
Ertapenem	160 ± 60	100	100	0.4 ± 0.05	0.13	0.2	0.0025	0.0013	0.002

[a]Kinetic parameters for OXA-48 are from Docquier et al. (28), except for those for benzylpenicillin, which are from Antonelli et al. (29), and cefotaxime-ceftazidime, which are from Poirel et al. (3).
[b]Kinetic parameters for OXA-181 are from Oueslati et al. (12).
[c]NH, no detectable hydrolysis.
[d]ND, not determined.

identical at the amino acid level, and their amino acid sequences were identical to the amino acid sequence with GenBank accession number WP_000050481.

The antimicrobial susceptibility of one representative transconjugant (AMA 1292) showed the expected profile according to the resistance genes present on pOXA436 (Table 2).

Characterization of OXA-436. Analysis of the OXA-436 amino acid sequence showed that, in contrast to the limited differences (≥97.4% amino acid sequence similarity) between previously described acquired OXA-48-like variants, the amino acid sequence of OXA-436 showed only 90.4 to 92.8% similarity to the amino acid sequences of the acquired OXA-48-like variants (Fig. 1). However, a greater amino acid sequence similarity (97.7 to 98.5%) to the amino acid sequences of bla_{OXA-48}-like genes from *Shewanella* isolates MR-4, MR-7, ANA-3, and JAB-1 (GenBank accession numbers CP000446, CP000444, CP000469, and CP022358, respectively) was observed (9). This may indicate that the *Shewanella* species that is the natural host of $bla_{OXA-436}$ is different from the *Shewanella* species that is the natural host of bla_{OXA-48} and $bla_{OXA-181}$, which has been identified to be *Shewanella xiamenensis* (9, 11). Subsequent cloning and expression of $bla_{OXA-436}$, bla_{OXA-48}, and $bla_{OXA-181}$ in *E. coli* TOP10 showed that OXA-436 produced the characteristic profile of OXA-48-like carbapenemases (12), resulting in a significant increase (>2-fold) in the MICs of penicillins and carbapenems and no significant change in the MICs of extended-spectrum cephalosporins and monobactams (aztreonam) (Table 2). The values of the kinetic parameters determined were also in broad agreement with the *in vitro* susceptibility data (Table 2) and were similar to the previously determined values of the kinetic parameters of OXA-48 (3, 28, 29) and OXA-181 (12) (Table 3). As shown for other OXA-48-like carbapenemases, the activity of OXA-436 against extended-spectrum cephalosporins was limited. Measurable activity of OXA-436 against ceftazidime (k_{cat}/K_m, 0.047 $\mu M^{-1} \cdot s^{-1}$) was observed, and this activity was slightly higher than that initially described for OXA-48 (k_{cat}/K_m, 0.001 $\mu M^{-1} \cdot s^{-1}$) (3), mainly due to the lower K_m. Activity against ceftazidime has been described for OXA-48-like variants, such as OXA-163 and OXA-232 (12). However, for OXA-163 and OXA-232, this has been at the expense of reduced activity against carbapenems and temocillin (OXA-232) (12). As the hydrolytic activity against ceftazidime did not result in a change in the MIC for *E. coli* TOP10 in the susceptibility assay (Table 2), this could indicate that the activity is observed only *in vitro* or that the *in vitro* activity is too low to contribute to activity *in vivo*. In contrast to previously published kinetic data, the kinetic assay for OXA-436 was performed in the presence of sodium bicarbonate, as CO_2 is required for *N*-carboxylation of the catalytic lysine (30). However, experiments without sodium bicarbonate showed similar results (data not shown), with measurable hydrolytic activity against ceftazidime being found. Investigation of the changes in the amino acid composition of OXA-436 in light of the three-dimensional structure

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of OXA-48 clearly showed that most mutations were at the protein surface, as would be expected for random mutations, because the inner core of enzymes is more conserved (31). The two buried residues Ile226 (similar to OXA-48 Val226) and Thr237 (Met237 in OXA-48), localized to the β7 sheet (28), are common residues in β sheets. Since the majority of the changes are localized at the surface in the three-dimensional structure of OXA-48, it seems likely that there would be no major changes to the overall fold of the OXA-436 structure compared to that of the OXA-48 structure. We believe that this is reflected in the similar functional characteristics of the two enzymes (Table 3). We are currently determining the crystal structure of OXA-436.

Conclusions. OXA-48 and OXA-48-like variants have become the dominant carbapenemases among *Enterobacteriaceae* in several regions and epidemiological settings (4). In this study, we identified a novel OXA-48-like variant named OXA-436 with higher amino acid sequence divergence from the currently identified OXA-48-like variants than the other OXA-48-like variants, whose sequences are closely related. Despite this divergence, OXA-436 shows broadly the same functional properties as other OXA-48-like variants with carbapenemase and penicillinase activity. The high degree of amino acid sequence similarity of OXA-436 with the amino acid sequences of OXA-48-like variants in members of the genus *Shewanella* whose species have so far not been determined further underlines the importance of species of this genus as the reservoir for OXA-48-like variants. The identification of $bla_{\text{OXA-436}}$ in three different *Enterobacteriaceae* species and four patients indicates the potential for further dissemination. The increased divergence of OXA-48-like variants complicates molecular-based detection and shows the importance of phenotypic and biochemical methods for the detection of carbapenemases.

MATERIALS AND METHODS

Bacterial strains. Six clinical isolates from four hospitalized patients were submitted to the Reference Laboratory for Antimicrobial Resistance and Staphylococci at the Statens Serum Institut in Copenhagen, Denmark, as part of the national surveillance for carbapenemase-producing organisms (approved by the Danish Data Protection Agency [no. 2015-57-0102]). Species identification was initially performed using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS; Bruker Daltonik, Bremen, Germany) and subsequently using the whole-genome sequence data described below. *Escherichia coli* TOP10 (Invitrogen, CA, USA) was used as the host strain for the cloning and expression of $bla_{\text{OXA-436}}$, $bla_{\text{OXA-48}}$, and $bla_{\text{OXA-181}}$. *E. coli* J53A3R was used for mating experiments. $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-181}}$ were obtained from previously described strains (32).

Antimicrobial susceptibility testing, phenotypic analysis, and initial PCR screening. MIC determinations were performed by broth microdilution using in-house-designed premade Sensititre microtiter plates (Trek Diagnostic Systems/Thermo Fisher Scientific, East Grinstead, UK). The MICs of the combination of temocillin (Eumedica, Basel, Switzerland) and meropenem (Hospira, Lake Forest, IL, USA) with avibactam (AstraZeneca, Cheshire, UK) were determined using the CLSI broth microdilution MIC methodology (33). Susceptibility patterns were interpreted according to the clinical breakpoints defined by EUCAST (version 7.1; http://www.eucast.org/clinical_breakpoints/). Combination gradient strips and discs for phenotypic detection of extended-spectrum β-lactamases (ESBLs; Becton Dickinson, Sparks, MD, USA/Liofilchem), AmpC β-lactamases (Rosco Diagnostica, Taastrup, Denmark/Liofilchem), and carbapenemases (Rosco Diagnostica) were performed according to the manufacturers' instructions. The Carba NP test was performed as previously described (34), and the OXA-48 *K*-SeT test (Coris BioConcept, Gembloux, Belgium) was performed according to the manufacturer's instructions. Initial PCR screening for carbapenemase-encoding genes was performed using assays previously described for the detection of bla_{VIM}, bla_{IMP}, bla_{GIM}, bla_{SPM}, bla_{SIM}, bla_{NDM}, bla_{KPC}, and $bla_{\text{OXA-48}}$ (3, 35, 36).

Whole-genome sequencing (WGS) and sequence analysis. Genomic DNA was extracted from carbapenemase-producing isolates using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Fragment libraries were constructed using a Nextera kit (Illumina, Little Chesterford, UK), followed by 251-bp paired-end sequencing (MiSeq; Illumina), according to the manufacturers' instructions. Paired-end sequence data were assembled using CLCbio's Genomics Workbench (version 8.0; Qiagen, Aarhus, Denmark).

The sequence reads from the *Citrobacter freundii* and *Enterobacter asburiae* isolates were aligned against the reference genomes of *C. freundii* CFNIH1 (GenBank accession number NZ_CP007557) and *E. asburiae* L1 (GenBank accession number NZ_CP007546), respectively, using the short-read alignment component of the Burrows-Wheeler aligner (BWA) (37). Using the Northern Arizona SNP Pipeline (http://tgennorth.github.io/NASP/), identification of SNP variants was performed using the GATK UnifiedGenotyper with filtering set to remove positions with less than 10× coverage and <90% unambiguous variant calls after positions within duplicated regions of the reference sequence were removed using the NUCmer program (38). Phylogenetic analysis of the identified SNPs was performed using

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maximum parsimony implemented in MEGA (version 6.0.6) software (39). Comparison of the sequences was performed using CLCbio's Genomics Workbench (version 8.0; Qiagen).

The web servers of ResFinder (version 2.1), PlasmidFinder (*Enterobacteriaceae*) (version 1.2), pMLST (IncHI2; version 1.4), and MLST (version 1.7) at http://www.genomicepidemiology.org/ were used to identify acquired antimicrobial resistance genes, plasmid replicon types, the plasmid multilocus sequence types (pMLST), and multilocus sequence types (MLST) from the assembled WGS data, respectively (16, 40, 41). For resistance genes, a threshold of 100% identity was used for the genes encoding β-lactamases, and 98% identity was used for all other genes. A lower identity threshold (60%) was initially used to search for putative novel β-lactamase genes.

Species identification using WGS data for the *Enterobacter* isolates was performed using phylogenetic analysis of fragments of the two housekeeping genes *rpoB* and *hsp60* as described by Paauw et al. (42). Comparisons of the sequences of contigs containing $bla_{\text{OXA-436}}$ were done using BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The amino acid sequences of acquired OXA-48-like variants and selected OXA-48-like variants were obtained from the NCBI Bacterial Antimicrobial Resistance Reference Gene Database (BioProject accession number PRJNA313047). Alignment and construction of a phylogenetic tree of OXA-48-like variants were done in the RAxML (version 8.2.8) program (43) using the MAFTT (version 7.305b) program (44) with a bootstrap of 100 replicates. A structural amino acid sequence alignment was created using the ESpript (version 3.0) program (http://espript.ibcp.fr/ESPript/ESPript/) (45).

A complete plasmid DNA sequence was obtained from a genome extracted from *E. asburiae* AMA 497 (Table 1) using a Genomic-tip 500/G kit (Qiagen, Hilden, Germany), which was used to prepare a 20-kb library for sequencing on a Pacific Biosciences RS II instrument using P6-C4 chemistry with a 360-min moving time on one single-molecule real-time (SMRT) cell (Pacific Biosciences, Menlo Park, CA) to generate 90,579 reads for a total of 1,503 Mb. Initial scaffolding was performed using HGAP (version 3) (SMRT analysis software, version 2.3.0; Pacific Biosciences) to give an average base coverage of 133×. The paired-end MiSeq data from AMA 497 described above were used for error correction and finalization of the sequencing data. The assembled plasmid sequence was annotated with the Prokka software tool (46) and manually curated for accuracy. A circular plasmid map was drawn using the CGView program (47).

Cloning, expression, and purification. For expression and purification of OXA-436, $bla_{\text{OXA-436}}$ was amplified using forward (5′-ATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGCGTGCGTTAGCCTTA-3′) and reverse (5′-CTTTGTTAGCAGCCTCGAATCACTAAGGAATAATTTTTTCCTGTTTCAG-3′) primers. The primers contain overlaps with pDEST17 (Invitrogen, CA, USA), and $bla_{\text{OXA-436}}$ with the signal peptide was cloned into pDEST17 using restriction-free cloning as previously described (48). The purified plasmid was transformed into *E. coli* BL21 Star(DE3)pRARE cells (49) and expressed in ZYP5052 (50) cultures containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. OXA-436 was isolated from the periplasm and purified by anion-exchange chromatography as described previously (51). The protein identity was confirmed by mass spectrometry.

For functional resistance profile analysis, cloning and expression of $bla_{\text{OXA-436}}$, $bla_{\text{OXA-48}}$, and $bla_{\text{OXA-181}}$ in an isogenic *E. coli* background were performed using a pCR-BluntII-TOPO cloning kit (Invitrogen, CA, USA). PCR fragments containing the entire sequences of the bla_{OXA} genes were obtained by PCR using Phusion high-fidelity PCR Mastermix with high-fidelity buffer (New England BioLabs, MA, USA) and the following primer pairs: OXAP18-15A (5′-TTTATTGCATTAGGCAAGGG-3′)/OXAP18-15B (5′-TACACAAATGC GCCGCTAACC-3′) for $bla_{\text{OXA-436}}$ and preOXA-48A/preOXA-48B for $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-181}}$ (13). The PCR products were run on a 1% agarose gel (Lonza, Rockland, ME, USA) and gel purified using a NucleoSpin gel and PCR cleanup kit (Macherey-Nagel, Düren, Germany) before cloning into the pCR-BluntII-TOPO vector and transformation into *E. coli* TOP10 according to the manufacturer's instruction (Invitrogen, CA, USA). Transformants were selected on Luria-Bertani (LB) agar plates (Becton Dickinson) containing 50 mg/liter kanamycin (Sigma-Aldrich, St. Louis, MO, USA). Verification of the cloned DNA insert was performed by PCR and Sanger sequencing (BigDye, version 3.1, technology; Applied Biosystems, CA, USA) using M13 forward (5′-GTAAAACGACGGCCAG-3′) and reverse (5′-CAGGAAACAGCTATGAC-3′) primers.

Kinetic analysis. All kinetic experiments for β-lactam substrates were performed using UV spectroscopy with a SpectraMax M2e spectrophotometer (Molecular Devices) at 25°C in triplicate in 100 mM sodium phosphate buffer (Sigma-Aldrich, St. Louis, MO, USA), pH 7.0, supplemented with 50 mM sodium bicarbonate (Merck Millipore, Darmstadt, Germany) and 0.2 mg/ml bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) (30). Hydrolysis measurements were performed with ampicillin ($\Delta\varepsilon_{235}$ = −820 M^{-1} cm^{-1}, 0.4 to 112.5 μM [Sigma-Aldrich]; 0.1 nM OXA-436), benzylpenicillin ($\Delta\varepsilon_{235}$ = −775 M^{-1} cm^{-1}, 222 to 3,000 μM [Panpharma]; 0.1 nM OXA-436), cefepime ($\Delta\varepsilon_{260}$ = −10,000 M^{-1} cm^{-1}, 14 to 462 μM [European Pharmacopoeia Reference Standards]; 1 nM OXA-436), cefotaxime ($\Delta\varepsilon_{260}$ = −7,500 M^{-1} cm^{-1}, 2.5 to 217.3 μM [Tokyo Chemical Industry Co., Ltd.]; 1 nM OXA-436), ceftazidime ($\Delta\varepsilon_{260}$ = −9,000 M^{-1} cm^{-1}, 3.9 to 500 μM [Sigma-Aldrich]; 1 nM OXA-436), ertapenem ($\Delta\varepsilon_{300}$ = −6,920 M^{-1} cm^{-1}, 7.8 to 1,000 μM [Merck Sharp & Dohme]; 5 nM OXA-436), imipenem ($\Delta\varepsilon_{300\ nm}$ = −9,000 M^{-1} cm^{-1}, 0.4 to 50 μM [European Pharmacopoeia Reference Standards]; 1 nM OXA-436), meropenem ($\Delta\varepsilon_{300}$ = −6,500 M^{-1} cm^{-1}, 0.4 to 50 μM [Sigma-Aldrich]; 5 nM OXA-436), and temocillin ($\Delta\varepsilon_{235}$ = −660 M^{-1} cm^{-1}, 3.9 to 500 μM [Eumedica Pharmaceuticals]; 5 nM OXA-436). Kinetic parameters were determined by nonlinear regression of the steady-state velocities as a function of the substrate concentrations using the Michaelis-Menten equation in Prism (version 6) software (GraphPad Software, CA, USA).

Design of $bla_{\text{OXA-436}}$ PCR. Primers for the specific PCR detection of a 672-bp $bla_{\text{OXA-436}}$ fragment, $bla_{\text{OXA-436}}$ forward (5′-TCGGCTGTGTTGATGGTGA-3′) and $bla_{\text{OXA-436}}$ reverse (5′-GTCATCCAGTTCGATCCA-3′), were designed using CLCbio's Main Workbench (version 7.5.1; Qiagen). A Qiagen multiplex PCR kit (Qiagen, Venlo, the Netherlands) was used for master mix preparation, and PCR conditions were initial

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heat activation at 94°C for 15 min and 26 cycles of denaturation for 30 s at 94°C, annealing for 90 s at 58°C, and elongation for 60 s at 72°C, followed by a final elongation step of 10 min at 72°C.

***In vitro* conjugation experiments.** *In vitro* conjugation experiments were performed using the sodium azide-resistant strain *E. coli* J53A3R as the recipient and *E. asburiae* AMA 497, *C. freundii* AMA 754, *K. pneumoniae* AMA 782, and *E. asburiae* AMA 810 as the donor strains. The recipient and donor strains were grown overnight in 50 ml Luria Bertani (LB) broth (with shaking at 120 rpm and 35°C). For the donor strains, LB broth was supplemented with 1 mg/liter ceftriaxone (Roche, Hvidovre, Denmark). After overnight incubation, 100 μl of the culture was added to 10 ml fresh LB broth and regrown without shaking at 35°C to a turbidity equivalent to that of an approximately 1.0 McFarland standard. Donor and recipient cells were subsequently mixed at a 1:1 ratio. Two hundred microliters of the mixed culture was added onto three separate 0.45-μm-pore-size nitrocellulose MF membrane filters (Merck Millipore Ltd., Cork, Ireland) placed on 5% blood agar plates (SSI Diagnostica, Hillerød, Denmark), and the plates were incubated overnight at 35°C. In addition, the mixed culture was left for liquid mating overnight at both room temperature and 35°C. After overnight incubation, the membrane filters were transferred to 5 ml 0.9% saline and vortexed. One hundred microliters of the 0.9% saline suspension with membrane filters and 100 μl of the resulting liquid mating culture were spread on LB agar plates supplemented with either 2 mg/liter ceftriaxone plus 100 mg/liter sodium azide (SSI Diagnostica, Hillerød, Denmark) or 8 mg/liter gentamicin (Sandoz, Holzkirchen, Germany) plus 100 mg/liter sodium azide. After overnight incubation at 35°C, putative transconjugant colonies were subcultured, and the successful transfer of $bla_{OXA-436}$ was subsequently confirmed by PCR as described above combined with species identification using MALDI-TOF MS (Bruker Daltonik). For confirmatory purposes, one representative transconjugant per donor-recipient combination was subjected to WGS analysis as described above.

Accession number(s). WGS data from the isolates have been deposited at the National Center for Biotechnology Information (NCBI) under BioProject PRJNA297498, and the annotated sequence of plasmid pOXA436 has been deposited in GenBank under accession number KY863418. Annotated contigs harboring $bla_{OXA-436}$ have been deposited in GenBank (GenBank accession numbers KT959103 to KT959108).

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We declare that we have no conflicts of interests.

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