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MECHANISMS OF RESISTANCE

Challenging Antimicrobial Susceptibility and Evolution of Resistance (OXA-681) during Treatment of a Long-Term Nosocomial Infection Caused by a *Pseudomonas aeruginosa* ST175 Clone

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ABSTRACT Selection of extended-spectrum mutations in narrow-spectrum oxacillinases (e.g., OXA-2 and OXA-10) is an emerging mechanism for development of *in vivo* resistance to ceftolozane-tazobactam and ceftazidime-avibactam in *Pseudomonas aeruginosa*. Detection of these challenging enzymes therefore seems essential to prevent clinical failure, but the complex phenotypic plasticity exhibited by this species may often lead to their underestimation. The underlying resistance mechanisms of two sequence type 175 (ST175) *P. aeruginosa* isolates showing multidrug-resistant phenotypes and recovered at early and late stages of a long-term nosocomial infection were evaluated. Whole-genome sequencing (WGS) was used to investigate resistance genomics, whereas molecular and biochemical methods were used for characterization of a novel extended-spectrum OXA-2 variant selected during therapy. The metallo-β-lactamase bla_{VIM-20} and the narrow-spectrum oxacillinase bla_{OXA-2} were present in both isolates, although they differed by an inactivating mutation in the *mexB* subunit, present only in the early isolate, and in a mutation in the bla_{OXA-2} β-lactamase, present only in the final isolate. The new OXA-2 variant, designated OXA-681, conferred elevated MICs of the novel cephalosporin–β-lactamase inhibitor combinations in a PAO1 background. Compared to OXA-2, kinetic parameters of the OXA-681 enzyme revealed a substantial increase in the hydrolysis of cephalosporins, including ceftolozane. We describe the emergence of the novel variant OXA-681 during treatment of a nosocomial infection caused by a *Pseudomonas aeruginosa* ST175 high-risk clone. The ability of OXA-681 to confer cross-resistance to ceftolozane-tazobactam and ceftazidime-avibactam together with the complex antimicrobial resistance profiles exhibited by the clinical strains harboring this new enzyme argue for maintaining active surveillance on emerging broad-spectrum resistance in *P. aeruginosa*.

KEYWORDS OXA, *Pseudomonas aeruginosa*, antimicrobial resistance, ceftazidime-avibactam, ceftolozane-tazobactam, class D beta-lactamase

The recent introduction into the clinical setting of the novel β-lactam–β-lactamase inhibitor combinations ceftolozane-tazobactam and ceftazidime-avibactam represents a step forward in the fight against β-lactam-resistant *Pseudomonas aeruginosa*

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infections because they show increased stability against the major mutation-driven β-lactam resistance mechanisms in this species, such as the overexpression of the chromosomal cephalosporinase AmpC and efflux pumps and the inactivation of the porin OprD (1). Nevertheless, *P. aeruginosa* is a constantly moving target, and the catalogue of reports documenting development of resistance to these new currently available drugs during therapy is growing. Overproduction and structural modification of AmpC have been identified as the main strategies developed by *P. aeruginosa* to compromise the activity of ceftolozane-tazobactam and ceftazidime-avibactam during therapy (2–4). Compared to AmpC, the contribution of class D OXA enzymes to the resistance profile of *P. aeruginosa* has been considered of less clinical importance, since narrow-spectrum oxacillinases (e.g., OXA-2 and OXA-10, which are relatively common in *P. aeruginosa* strains worldwide) exhibit only a very limited substrate profile, and extended-spectrum variants (ES-OXAs) are rarely reported (5, 6). However, recent *in vivo* studies have evidenced that, like AmpC enzymes, they are prone to developing extended-spectrum mutations during the course of therapy, resulting in derivatives able to confer cross-resistance to ceftolozane-tazobactam and ceftazidime-avibactam (7). It is also worth mentioning that the vast majority of these enzymes are frequently mobilized in transposable elements within class 1 integrons and cotransferred with aminoglycoside-modifying enzymes or other remarkable resistance determinants, such as VIM-type metallo-β-lactamases (MBLs), the acquisition of which restricts the choice of therapy to an alarming extent (8). The overwhelming association of these horizontally acquired enzymes with a few epidemic multidrug-resistant (MDR)/extensively drug-resistant (XDR) *P. aeruginosa* clones disseminated across hospitals worldwide, the so-called high-risk clones, further adds to the concern (9).

Given this background, whereas studies are being conducted to identify potential strategies to prevent AmpC-mediated β-lactam resistance, such as inactivation of the key AmpG permease involved in AmpC overexpression (10), active surveillance is required to detect strains harboring these challenging horizontally acquired class D OXA β-lactamases and thus contribute to extending the life of these new currently available drugs. However, this is not always straightforward, since *P. aeruginosa* is equipped with a considerable repertoire of chromosomal genes able to modulate its levels of antimicrobial resistance. As a result, *P. aeruginosa* exhibits an outstanding phenotypic plasticity that severely hampers the inference of the underlying resistance mechanisms, which can often therefore be underestimated and lead to inadequate treatment, the development of resistance, and clinical failure (11). Here, we dissect the underlying resistance mechanisms of two sequence type 175 (ST175) *P. aeruginosa* isolates recovered at the early and late stages of a long-term nosocomial infection. A whole-genome sequencing (WGS) approach was used to depict the mutational and transferable resistance mechanisms involved, whereas molecular and biochemical studies were performed to characterize a novel OXA-2 variant that emerged during the course of treatment.

RESULTS AND DISCUSSION

Molecular typing, antimicrobial susceptibility, and genomic features of the clinical isolates. Molecular typing procedures revealed that PA34 and PA145 were derived from the same progenitor strain that colonized the patient, who received treatment with several antipseudomonal β-lactams. Both isolates yielded identical SpeI pulsed-field gel electrophoresis (PFGE) genotype patterns and were assigned by multilocus sequence typing (MLST) to the epidemic ST175 high-risk clone, which is highly prevalent in multiple European hospitals, particularly in Spain and France. The antimicrobial susceptibility results and main mutational and horizontally acquired resistance determinants for strains PA34 and PA145 are summarized in Table 1. The two isolates demonstrated similar MICs of aminoglycosides, quinolones, colistin, and carbapenems, although major differences were observed for the rest of the β-lactams. Indeed, PA34 showed wide susceptibility to piperacillin-tazobactam, aztreonam, ceftazidime, ceftazidime-avibactam, and cefepime but yielded an unexpected ceftolozane-tazobactam MIC of

TABLE 1 Strain, sequence type, antimicrobial susceptibility, and main mutational and transferable resistance mechanisms exhibited by isolates PA34 and PA145[a]

		MIC (μg/ml)[b]													Resistance mechanisms [gene(s) (mutations)]						
															Chromosomal					Transferable	
Strain	ST	TIC (S ≤ 16)	P/T (S ≤ 16)	AZT (S ≤ 16)	CAZ (S ≤ 8)	CTZ (S ≤ 4)	CZA (S ≤ 8)	FEP (S ≤ 8)	TOB (S ≤ 4)	AK (S ≤ 8)	IPM (S ≤ 4)	MRP (S ≤ 2)	CIP (S ≤ 0.5)	COL (S ≤ 2)	MexAB-OprM	MexXY-OprM	AmpC	OprD	QRDR	β-Lactamases	AMEs
PA34	175	256	<4/4	0,19	4	16/4	4/4	4	>32	32	64	16	16	1	*mexB* ($nt_{80}\Delta10$)	*mexX* (W358R), *mexZ* (G195D)		nt_{49}InsGCACT	*gyrA* (T83I, D87N), *parC* (S87W)	bla_{OXA-2}, bla_{VIM-20}	*aadB*, *aac(6′)-Ib3*, *aphA-6*, *aadA13*
PA145	175	>512	32/4	4	64	>32/4	32/4	16	>32	32	32	32	>16	1		*mexX* (W358R), *MexZ* (G195D)		nt_{49}InsGCACT	*gyrA* (T83I, D87N), *parC* (S87W)	$bla_{OXA-681}$, bla_{VIM-20}	*aadB*, *aac(6′)-Ib3*, *aphA-6*, *aadA13*

[a]ST, sequence type; S, sensitivity breakpoint; TIC, ticarcillin; P/T, piperacillin-tazobactam; AZT, aztreonam; CAZ, ceftazidime; CTZ, ceftolozane-tazobactam; CZA, ceftazidime-avibactam; FEP, cefepime; TOB, tobramycin; AK, amikacin; IPM, imipenem; MRP, meropenem; CIP, ciprofloxacin; COL, colistin; QRDR, quinolone resistance-determining region; AMEs, aminoglycoside-modifying enzymes.
[b]2019 EUCAST breakpoints.

16 μg/ml. In contrast, PA145, which was isolated 70 days later after several courses of antimicrobial therapy with piperacillin-tazobactam, cefepime, and ceftazidime, exhibited an XDR phenotype and remained susceptible to only aztreonam and colistin. WGS analysis identified that both isolates shared the classical MexZ mutation (G195E) involved in MexXY overexpression and the triple quinolone resistance-determining region (QRDR) mutations affecting GyrA (T83I and D87N) and ParC (S87W), hallmarks of the mutational resistome of this high-risk clone (12), but lacked other characteristic mutations described in Spanish and French ST175 isolates, such as those affecting AmpR (G154R), the LysR-type transcriptional regulator of AmpC, responsible for AmpC overexpression, and the OprD Q142X inactivating mutation, which was replaced by a 5-bp insertion that also resulted in a truncated OprD. Mutations previously associated with resistance were not identified in the genes coding for the penicillin-binding proteins (PBP1a, PBP1b, PBP2, PBP3, or PBP4), suggesting that β-lactam-binding affinities were not altered in the two isolates.

Analysis of horizontally acquired resistance determinants revealed the presence of several genes encoding aminoglycoside-modifying enzymes, including *aadB*, which is frequently detected in this lineage, *aadA13*, *aac(6′)-Ib3*, and *aphA-6*, which explains the high MICs observed for aminoglycosides. Likewise, analysis of horizontally acquired β-lactamases identified the presence of the genes coding for the $bla_{\text{VIM-20}}$ carbapenemase (VIM-2 cluster) and the narrow-spectrum oxacillinase $bla_{\text{OXA-2}}$ in both isolates. These results were partially unexpected, since although the presence of these β-lactamases correlated well with the phenotype exhibited by the final XDR isolate PA145 (susceptible to only colistin and aztreonam), it failed to explain the paradoxical resistance to ceftolozane-tazobactam with respect to susceptibility to piperacillin-tazobactam (MIC = 4/4 μg/ml), ceftazidime (MIC = 4 μg/ml), and cefepime (MIC = 4 μg/ml) of the early isolate PA34.

However, an analysis of the genes coding for the MexAB-OprM transporter, which strongly contributes to *P. aeruginosa*'s natural resistance to multiple β-lactams (13), revealed the presence of a 10-bp mutational insertion in the *mexB* gene, leading to a functional loss of MexAB-OprM pumping activity. Abolition of MexAB-OprM efflux explains the particularly low MIC of aztreonam (0.19 μg/ml), which is not hydrolyzed by either VIM-20 or OXA-2 enzymes (14, 15), but also seems to compensate for the effect of carbapenemase production on the MICs of ceftazidime and cefepime, since both are also substrates for this pump and exhibit MICs below the clinical breakpoints (4 μg/ml each). Indeed, a similar effect of the latter has been described in ceftazidime-susceptible cystic fibrosis isolates with deficient MexAB-OprM efflux and stable AmpC overproduction (16). On the other hand, it remains unclear why ceftolozane-tazobactam MICs remained above the clinical breakpoints (16 μg/ml) in this early strain, since this antibiotic bypasses the main intrinsic and mutational resistance mechanisms exhibited by *P. aeruginosa* and tends to show increased activity compared to other antipseudomonal cephalosporins against both wild-type and resistant strains. A possible explanation is a higher hydrolytic activity of VIM-20 against ceftolozane than against ceftazidime and cefepime, although the precise mechanisms that sustain this complicated resistance phenotype cannot be totally solved using the data presented here and deserve further investigation in future specific studies.

Moreover, it also deserves mention that the deficient MexAB-OprM efflux found in the isolate PA34 indicates that the late isolate PA145 is not the result of the direct evolution of the former, since it is difficult to conceive that the MexAB-OprM system could recover its functionality despite a 10-bp insertion in the *mexB* gene. Certainly, this was not unexpected, since in patients with chronic respiratory infections, as in this case, it is frequently found that many populations of *P. aeruginosa* coexist in the lung, and the timing and order of their recovery do not guarantee the evolutionary trajectory of the isolates. However, taking into account the genomic features described above and the results obtained by molecular typing procedures, it is evident that both isolates were derived from the same VIM-20/OXA-2-coproducing ST175 progenitor strain.

TABLE 2 MICs for the PAO1 transformants producing OXA-2 and OXA-681 β-lactamases[a]

Strain	MIC (μg/ml)									
	TIC	TZP	AZT	CAZ	CZA	CTO	CTZ	FEP	IPM	MRP
PAO1(pUCP24)	16	≤4/4	4	≤1	≤1	≤0.5	≤0.5/4	≤1	1	≤0.5
PAO1(pUCP-OXA-2)	256	≤4/4	4	4	1/4	0.5	0.5/4	2	2	8
PAO1(pUCP-OXA-681)	256	≤4/4	4	64	32/4	32	32/4	4	2	2

[a]TIC, ticarcillin; TZP, piperacillin-tazobactam; AZT, aztreonam; CAZ, ceftazidime; CZA, ceftazidime-avibactam; CTO, ceftolozane; CTZ, ceftolozane-tazobactam; FEP, cefepime; IPM, imipenem; MRP, meropenem.

Characterization of β-lactamases. Analysis of the sequences of acquired β-lactamases showed that isolate PA145 harbored a previously undescribed 3-bp (TAG) deletion affecting two codons in the bla_{OXA-2} gene, leading to the deletion of isoleucine at position 159 and the replacement of glutamic acid by lysine at position 160. This novel OXA-2 variant was designated OXA-681. To our knowledge, this is the first report in which an extended-spectrum OXA β-lactamase is generated from a narrow-spectrum OXA β-lactamase through the concomitant deletion and substitution of two conserved residues. Remarkably, both changes were located within the Trp-X-Glu-X-X-Leu-X-Ile-Ser stretch, a conserved motif in class D β-lactamases, also called the omega loop, which plays a major role in the accommodation of the β-lactam molecule in the active site (17–19). Based on the crystal structure of the OXA-2 enzyme available (PDB accession number 1K38), it is expected that the change of a negatively charged residue (E160) to a positively charged residue (K160), as well as the deletion of a neutral group (I159), would change significantly the charge of this region of the enzyme. As a consequence, this change might alter the binding mode of cephalosporins, as a result of trying to accommodate the side-chain groups of the ligand in a more positive environment.

In order to evaluate the impact of these substitutions on β-lactam resistance, bla_{OXA-2} and $bla_{OXA-681}$ genes were cloned in parallel and expressed in a PAO1 background. Comparative MIC data for PAO1 expressing OXA-2 and OXA-681 are detailed in Table 2. Compared to OXA-2, the production of OXA-681 caused a 16-fold increase in the MIC of ceftazidime, a 32-fold increase in the MIC of ceftazidime-avibactam, and a 64-fold increase in the MIC of ceftolozane and ceftolozane-tazobactam, providing good evidence of the increased spectrum of resistance to antipseudomonal cephalosporins conferred by this new OXA-2 variant. In contrast, the production of OXA-681 caused a 4-fold decrease in the MIC of meropenem. Interestingly, the antimicrobial susceptibility profile conferred by OXA-681 on a PAO1 background is almost identical to the one recently reported for OXA-539, an OXA-2-derived extended-spectrum cephalosporinase selected during ceftazidime therapy (7). Therefore, the emergence of OXA-681 adds further evidence of the worrying effect of the classic antipseudomonal cephalosporins on the selection of class D β-lactamase variants with increased activity against the novel β-lactam–β-lactamase inhibitor combinations ceftolozane-tazobactam and ceftazidime-avibactam.

Genetic context of β-lactamases. Both the bla_{OXA-2} and $bla_{OXA-681}$ genes were documented to be located in a 6.625-kb class 1 integron harboring the bla_{VIM-20}, *aac(6′)-Ib3*, $bla_{OXA-2/681}$, and *orfX* (putative exonuclease) gene cassettes and truncated at its right-hand extremity by the conserved linear organization of IS*Aba14*–*aphA(3′)-VI* (20) (Fig. 1). All attempts to transfer OXA-2/OXA-681 through conjugation and electroporation yielded persistently negative results. However, an analysis of the whole-genome sequencing data using plasmidSPAdes software identified the integron containing the bla_{OXA-2} and $bla_{OXA-681}$ genes in a plasmid contig, therefore suggesting the plasmid location of the β-lactamases.

Relative kinetic properties of OXA-681 and OXA-2. Kinetic measurements were performed on OXA-2 and OXA-681. Consistent with the MIC data, kinetic assays with ceftazidime, ceftolozane, and meropenem revealed major differences between the two proteins (Table 3). As shown, OXA-681 exhibited an enhanced ability to hydrolyze

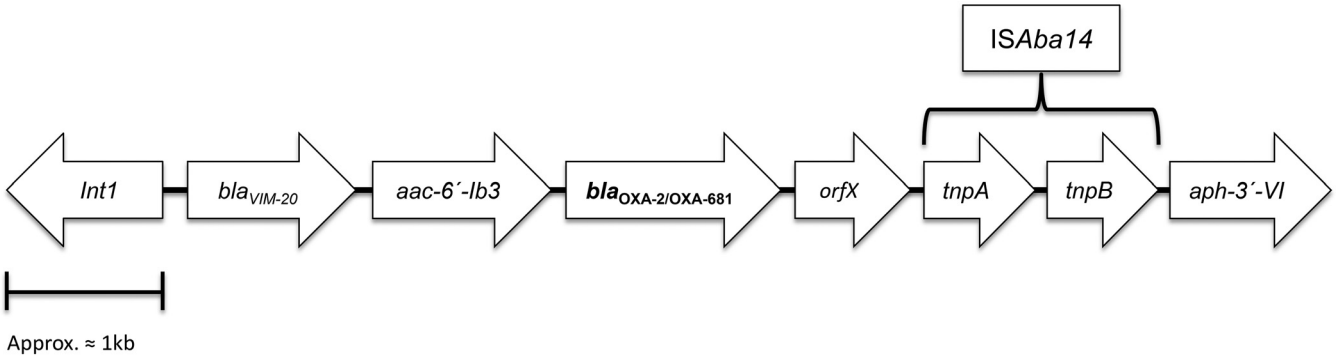

FIG 1 Schematic structure of the truncated integron harboring OXA-2 and OXA-681 β-lactamases.

cephalosporins compared to OXA-2. The key amino acid changes I159del and E160K on the OXA-2 protein caused a substantial enhancement of the enzyme-substrate binding affinity (K_m) without significantly affecting turnover rates (k_{cat}). This effect is noticeable for both ceftazidime and ceftolozane, yielding k_{cat}/K_m (catalytic efficiency) ratios 1,514- and 17-fold higher than those for OXA-2. It is tempting to suggest, therefore, that the cephalosporin sequestration conferred by OXA-681 in the *P. aeruginosa* background (characterized by high impermeability) may play a greater role in resistance than turnover rates (21). In contrast, OXA-681 exhibited lower turnover rates and catalytic efficiency toward meropenem, illustrating that its newly evolved activity against cephalosporins is detrimental to the natural carbapenemase activity of the parental OXA-2 protein. On the other hand, the 50% inhibitory concentration (IC_{50}) values (Table 4) show that the I159del and E160K mutations present in the OXA-681 enzyme confer not only increased cephalosporin hydrolysis but also a slight, ~10-fold decrease in the affinity for tazobactam and the novel diazabicyclooctane (DBO) inhibitor avibactam compared to OXA-2.

Concluding remarks. The new antipseudomonal agents ceftolozane-tazobactam and ceftazidime-avibactam, which are now available, are increasingly recognized as the most suitable options for the treatment of MDR *P. aeruginosa* infections, and preserving their clinical utility represents a major challenge for the medical community. Although resistance to these new antibiotics in *P. aeruginosa* emerges mainly through specific mutations leading to the overexpression and structural modification of AmpC (22, 23), the selection of extended-spectrum mutations from narrow-spectrum OXA-type β-lactamases, such as OXA-2, represents another important emerging resistance mechanism. Here, we add evidence of this importance in the characterization of the tight-binding OXA-681 enzyme, which exhibits specific amino acid substitutions and abrogates the utility of the novel cephalosporin–β-lactamase inhibitor combinations. The relevance of these results is emphasized first by the fact that this enzyme emerged in a strain belonging to the epidemic ST175 high-risk clone, which may further disseminate among patients, and second by the fact that OXA-681 was located in a plasmid-encoded class 1 integron coharboring the MBL VIM-20, resulting in a challenging MBL/ES-OXA MDR element that could be disseminated further among strains.

TABLE 3 Kinetic parameters of the β-lactamases OXA-2 and OXA-681 for representative substrates[a]

	OXA-2			OXA-681			
Drug	K_m (μM) ± SD	k_{cat} (s^{-1}) ± SD	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (μM) ± SD	k_{cat} (s^{-1}) ± SD	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat}/K_m ratio for OXA-681/OXA-2
Nitrocefin	56.9415 ± 14.5405	128.7833 ± 12.4233	2,261	78.0759 ± 19.0628	11.0857 ± 2.0566	141.9862	0.06
Ceftazidime	991.2681 ± 175.1077	0.0025 ± 0.0002	0.0025	0.6706 ± 0.2037	0.0025 ± 0.0007	3.7280	1,514
Ceftolozane	305.2738 ± 35.4224	0.0003 ± 0.0001	0.0009	31.9304 ± 7.9682	0.0005 ± 0.0001	0.0157	17
Meropenem	0.0384 ± 0.0052	0.0097 ± 0.0006	252.6042	0.0036 ± 0.0006	0.0004 ± 0.0001	111.1111	0.40

[a]Data represent the means from three independent experiments.

TABLE 4 IC_{50} parameters of the β-lactamase inhibitors tazobactam and avibactam against OXA-2 and OXA-681[a]

Drug	IC_{50} (nM) ± SD		Ratio of IC_{50} of OXA-681/IC_{50} of OXA-2
	OXA-2	OXA-681	
Tazobactam	1.48 ± 0.4761	13 ± 1.8302	9
Avibactam	367 ± 24.1150	3,050 ± 902.4786	8

[a]Data represent the means from three independent experiments.

Finally, the impaired MexAB-OprM efflux identified in the early isolate of this work, which is a hallmark of strains recovered from long-term infections such as cystic fibrosis or bronchiectasis and is expected to play a minor role in strains recovered from acute infections, appears as a mechanism that could contribute to the emergence of complex resistance phenotypes that make it difficult to infer the relevant underlying resistance mechanisms in *P. aeruginosa* and therefore deserves further investigation. Altogether, these findings argue for the need for maintaining active surveillance on emerging broad-spectrum resistance in MDR *P. aeruginosa* strains.

MATERIALS AND METHODS

Case summary and clinical strains. An 82-year-old patient with bronchiectasis and chronic respiratory failure, with a history of multiple hospital admissions during the preceding months, presented to the emergency department of a tertiary-level academic hospital with severe dyspnea, cough with sputum production, and oxygen desaturation on room air requiring noninvasive ventilatory support. The patient was receiving chronic respiratory treatment with tobramycin and azithromycin. Previous microbiological studies revealed positive respiratory cultures for several nonfermenting Gram-negative bacilli, including *P. aeruginosa*, *Achromobacter xylosoxidans*, and *Stenotrophomonas maltophilia*. After obtaining sputum samples and starting empirical therapy with piperacillin-tazobactam and trimethoprim-sulfamethoxazole (T/S), the patient was admitted to the pneumology department for follow-up. Forty-eight hours later, the sputum sample obtained upon admission grew MDR *P. aeruginosa* exhibiting an unusual susceptibility profile, being resistant to ceftolozane-tazobactam but susceptible to piperacillin-tazobactam, ceftazidime, ceftazidime-avibactam, cefepime, aztreonam, and colistin (PA34). The patient remained on therapy with piperacillin-tazobactam, and the cough and sputum disappeared. On hospital day 14, piperacillin-tazobactam was discontinued. Three days later, the patient experienced a new episode of severe cough and sputum production. Sputum samples yielded MDR *P. aeruginosa* with the same susceptibility pattern as the one obtained at admission (PA34), which was treated with cefepime for 29 days. After this time, the patient's condition alternated between brief periods (3 to 4 days) of clinical improvement and severe acute exacerbations, yielding sputum cultures positive for *A. xylosoxidans* and *P. aeruginosa* that were treated for 10 days with T/S and for 15 days with ceftazidime, respectively. At the start of a novel acute exacerbation on hospital day 77, respiratory cultures grew XDR *P. aeruginosa* susceptible to only aztreonam and colistin (PA145). Therapy with aztreonam was continued and colistin inhalation was maintained, but the respiratory condition of the patient deteriorated, and he died a few days later.

Antimicrobial susceptibility testing. MICs of ticarcillin, piperacillin-tazobactam, ceftazidime, ceftazidime plus 4 μg/ml avibactam, cefepime, aztreonam, ceftolozane, ceftolozane plus 4 μg/ml tazobactam, imipenem, meropenem, tobramycin, amikacin, ciprofloxacin, and colistin were determined by broth microdilution according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) v.9.0 clinical breakpoints and guidelines (http://www.eucast.org/clinical_breakpoints/).

Molecular typing. Clonal relatedness was assessed by using SpeI pulsed-field gel electrophoresis (PFGE) according to protocols described previously (24). Multilocus sequence typing was performed according to the scheme proposed by Curran et al. (25). The isolates were assigned a sequence type (ST) according to the allelic profiles available in the MLST Database (http://pubmlst.org/paeruginosa).

Determination of resistance mechanisms by WGS. The presence of mutations within 146 chromosomal genes involved in the modulation of antimicrobial resistance was determined as described previously (26). Indexed paired-end libraries were generated from genomic DNA using a commercial library preparation kit (Nextera XT DNA library preparation kit; Illumina) and sequenced on an Illumina MiSeq benchtop sequencer with a MiSeq reagent kit (version 3; Illumina Inc., USA). The resulting paired-ended reads were compared to the *P. aeruginosa* PAO1 reference genome, and sequence variation was further analyzed for the 146 chromosomal genes related to antimicrobial resistance. The presence of horizontally acquired resistance determinants was also evaluated using online databases (https://cge.cbs.dtu.dk//services/ResFinder/).

Characterization of β-lactamases. bla_{OXA-2} and $bla_{OXA-681}$ genes were amplified in parallel using the primer pair OXA-2-F-Xba1 (5′-TCCTCTAGAGTTGGGCATTAGGAAAAG-3′) and OXA-2-R-HindIII (5′-TCCAA GGCTTTTATCGCGCAGCGTCCGAG-3′). The purified PCR products were digested with XbaI and HindIII, ligated into pUCP24, and transformed into *Escherichia coli* XL1-Blue. The resulting transformants were selected in LB agar plates containing 5 μg/ml gentamicin–X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-IPTG (isopropyl-β-D-thiogalactopyranoside). Recombinant plasmids were electropo-

rated into reference strain PAO1 and plated in LB agar plates containing 30 μg/ml gentamicin. MICs of ticarcillin, piperacillin-tazobactam, ceftazidime, ceftazidime-avibactam, cefepime, aztreonam, ceftolozane, ceftolozane-tazobactam, imipenem, and meropenem for the resulting PAO1 transformants were determined according to the methodology described above.

Characterization of genetic elements harboring acquired β-lactamases. The possible plasmid location of acquired β-lactamase genes was preliminarily analyzed through transformation and conjugation experiments according to previously described protocols (27). Moreover, the plasmid location of acquired β-lactamase genes was further evaluated from whole-genome sequencing data containing short Illumina reads using the plasmidSPAdes software tool (28). Sequence analysis of the integron regions harboring bla_{VIM-20} and $bla_{OXA-2/OXA-681}$ genes scattered in different contigs was performed by PCR followed by sequencing.

Protein purification. bla_{OXA-2} and $bla_{OXA-681}$ genes were directionally cloned into the p-GEX-6P-1 plasmid (GE Healthcare, Little Chalfont, UK) for protein purification. Genes were amplified using the primer pair OXA-2-F-BamHI (5′-CGCGGATCCGCGCAAGAAGGCACG-3′) and OXA-2-R-SmaI (5′-TCCCCCGG GGGATTATCGCGCAGC-3′), digested with BamHI and SmaI, and ligated into BamHI/SmaI-digested p-GEX-6P-1. The recombinant plasmids were electroporated into the protease-deficient *E. coli* BL21 strain to generate two fusion proteins: glutathione *S*-transferase (GST)/OXA-2 and GST/OXA-681. The GST tag was then cleaved off, and the resulting proteins were then purified to homogeneity using the GST gene fusion system (Amersham Pharmacia Biotech, Europe) according to the manufacturer's instructions. Finally, SDS-PAGE was performed to ascertain the absence of impurities in the final extract (≥95% purity).

Kinetic assays. The kinetic parameters of purified OXA-2 and OXA-681 β-lactamases were determined at room temperature using a Nicolet Evolution 300 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) or an Epoch 2 microplate spectrophotometer (BioTek, VT, USA). Each experiment was performed in triplicate using 50 mM sodium phosphate with 20 mM sodium bicarbonate in 0.2-cm-path-length cuvettes. Kinetic parameters were determined by measuring the initial hydrolysis rates and using a Hanes-Woolf linearization of the Henri-Michaelis-Menten equation. For each antibiotic, the K_m values were measured as the K_i by measuring the residual β-lactamase activity, using nitrocefin (Oxoid, Hampshire, United Kingdom) as the reporter substrate, whereas k_{cat} values were determined by monitoring the direct hydrolysis of the antibiotic at a substrate concentration much higher than the K_m. For inhibition kinetics, the IC_{50}s for tazobactam and avibactam were calculated as the inhibitory concentrations resulting in a 50% reduction of nitrocefin hydrolysis and were determined after a 10-min preincubation with the enzyme at 490 nm. The wavelengths and absorption coefficients used for each antibiotic were as follows: 260 nm/Λ ε of $-8{,}660$ M^{-1} cm^{-1} for ceftazidime, 254 nm/Λ ε of $-6{,}810$ M^{-1} cm^{-1} for ceftolozane, and 297 nm/Λ ε of $-10{,}940$ M^{-1} cm^{-1} for meropenem.

Accession number(s). The nucleotide sequences described in this work have been deposited in the GenBank database under the following accession numbers: MH986647.1 ($bla_{OXA-681}$), SRX5389645 (genome sequence data for strain PA34), and SRX5389644 (genome sequence data for strain PA145).

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We have no conflicts of interest to declare.

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