AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

MECHANISMS OF RESISTANCE

Carbapenem Resistance Caused by High-Level Expression of OXA-663 β-Lactamase in an OmpK36-Deficient *Klebsiella pneumoniae* Clinical Isolate

Peijun Ma,[a,b,c] Hannah H. Laibinis,[a,b] Christoph M. Ernst,[a,b,c] Deborah T. Hung[a,b,c]

[a]The Broad Institute of MIT and Harvard, Cambridge, Massachusetts, USA

[b]Department of Molecular Biology, Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston, Massachusetts, USA

[c]Department of Genetics, Harvard Medical School, Boston, Massachusetts, USA

ABSTRACT Carbapenem resistance is mainly mediated by carbapenemases or extended-spectrum β-lactamases (ESBL) plus a loss of porins. However, we have identified a *Klebsiella pneumoniae* clinical isolate that contains neither carbapenemases nor ESBLs. Instead, we found that high-level expression of a novel bla_{OXA-10}-derived β-lactamase gene, $bla_{OXA-663}$, in conjunction with OmpK36 deficiency results in high-level carbapenem resistance. This finding demonstrates the combinatorial complexity of factors, including β-lactamase activity, its expression levels, and porin activity, that yield carbapenem resistance.

KEYWORDS *Klebsiella*, OXA-663, β-lactamases, carbapenem resistance

Resistance to β-lactam antibiotics emerged even before the first β-lactam, the microbial metabolite penicillin, was used therapeutically (1), with many bacteria naturally harboring β-lactamases. Each deployment of the next, new, more powerful β-lactam and/or β-lactamase inhibitor has been followed by the inevitable emergence of resistance. This cycle was recently observed for the extended-spectrum β-lactams with the emergence and expansion of extended-spectrum β-lactamases (ESBLs) (2) and even more recently for the carbapenems (2, 3). Carbapenem resistance is mainly mediated by the production of carbapenemases, such as class A serine β-lactamases (i.e., KPC family), class B metallo-β-lactamases (i.e., NDM, IMP, and VIM), and class D serine β-lactamases (i.e., OXA-48) (2, 3). Carbapenem resistance can also occur via a combination of an ESBL and a malfunctioning porin where an ESBL alone is not sufficient to cause resistance (1, 2).

Previously, our colleagues sequenced 122 carbapenem-resistant *Enterobacteriaceae* (CRE) isolates that were obtained from four large referral hospitals in the United States, aiming to investigate the diversity of the genetic mechanisms of carbapenem resistance (4). Whole-genome sequencing (WGS) revealed that the majority of these isolates contain either carbapenemase genes or ESBL genes in combination with porin gene mutations. However, there were 15 isolates (12%) in which none of the known carbapenem resistance mechanisms were identified (4). Among these 15 isolates, only 2 isolates had high-level resistance to carbapenems (MIC > 16 μg/ml), including one *Klebsiella pneumoniae* strain, BIDMC35 (NCBI accession no. PRJNA202047). In this study, we investigated the carbapenem-resistance mechanism of BIDMC35.

While WGS did not detect any known carbapenemase or ESBL genes in BIDMC35, it did identify a bla_{OXA-10} variant gene that resides on an IncA/C2 plasmid. Besides this bla_{OXA-10} variant gene, the only β-lactamase gene identified in BIDMC35 is the chromosome-encoded bla_{SHV-1}. In comparison to the original bla_{OXA-10}, this bla_{OXA-10} variant gene harbors a point mutation that results in a T16K substitution (4, 5); this allele is designated

Received 15 June 2018 **Returned for modification** 13 July 2018 **Accepted** 10 August 2018

Accepted manuscript posted online 20 August 2018

Citation Ma P, Laibinis HH, Ernst CM, Hung DT. 2018. Carbapenem resistance caused by high-level expression of OXA-663 β-lactamase in an OmpK36-deficient *Klebsiella pneumoniae* clinical isolate. Antimicrob Agents Chemother 62:e01281-18. https://doi.org/10.1128/AAC.01281-18.

Address correspondence to Deborah T. Hung, hung@molbio.mgh.harvard.edu.

TABLE 1 Plasmids used in the study

Plasmid	Description	Reference or source
pBAD24	Expression vector containing arabinose inducible P_{BAD} promoter, Amp^r	8
pSMART-LCKan	Cloning vector without a promoter, Kan^r	Lucigen
pRU1097	Expression vector containing Gm^r resistance gene, Gm^r	15
pBAD24_Gm	Gm^r resistance gene was amplified by PCR from pRU1097 and then ligated to the HindIII site of pBAD24, Gm^r	This study
pBAD24_Gm_$bla_{OXA-663}$	$bla_{OXA-663}$ was amplified from BIDMC35 and ligated to pBAD24_Gm in the EcoRI-KpnI sites, Gm^r	This study
pBAD24_Gm_bla_{OXA-10}	$bla_{OXA-663}$ in pBAD24_Gm_$bla_{OXA-663}$ was site-mutagenized to bla_{OXA-10}, Gm^r	This study
pSMART_$bla_{OXA-663}$	$bla_{OXA-663}$ and the 182-bp upstream region ($P_{OXA-10}{}^c$) were amplified from BIDMC35 and blunt ligated to pSMART-LCKan, Kan^r	This study
pSMART_Gm_*ompK36*	*ompK36* containing the promoter region was amplified from UCI64 and blunt ligated to pSMART-LCKan. Gm^r resistance gene was amplified by PCR from pRU1097 and then ligated to the XbaI site, Gm^r	This study

$bla_{OXA-663}$. To date, the class D OXA-10 β-lactamase has been predominantly isolated from *Pseudomonas aeruginosa* and has been classically thought of as a narrow-spectrum β-lactamase (1). The breadth of its spectrum was revisited by the demonstration of its ability to confer resistance to carbapenems when expressed in *Acinetobacter baumannii* but not in *Escherichia coli* (6). Neither bla_{SHV-1} nor bla_{OXA-10} has to date been shown to confer carbapenem resistance in *K. pneumoniae*. Because some OXA-type ESBL genes have been reported that differ from bla_{OXA-10} by just a few mutations (1), we hypothesized that the novel mutation observed in $bla_{OXA-663}$ might play a role in its high-level carbapenem resistance, despite the fact that none of the reported OXA-10-derived ESBLs harbor this particular amino acid substitution and that this mutation is located in the leader signal sequence (5). In addition to the single base pair substitution in the coding region, the upstream region of $bla_{OXA-663}$ differs from those of bla_{OXA-10} genes identified in *P. aeruginosa* and in other *K. pneumoniae* strains (i.e., MGH71 and MGH65). While all contain a 182-nucleotide consensus upstream region ($P_{OXA-10}{}^c$), BIDMC35 contains an additional 174 nucleotides upstream of the consensus sequence ($P_{OXA-663}$; 356 bp total) compared to the upstream regions of MGH71 and MGH65. Compared to the upstream sequences of bla_{OXA-10} in some *P. aeruginosa* strains (7), the 174-bp region of $P_{OXA-663}$ contains a 34-bp duplication and a 5-bp insertion.

We first investigated if $bla_{OXA-663}$ can confer resistance to carbapenems. We expressed $bla_{OXA-663}$ in a wild-type *E. coli* strain 10beta (NEB) using the pSMART-LCKan (Lucigen) plasmid (Table 1). When we tried to express $bla_{OXA-663}$ by including the whole upstream region ($P_{OXA-663}$) along with the coding sequence, we were unable to obtain any transformants, suggesting that *E. coli* may not tolerate this DNA construct. However, we were able to express $bla_{OXA-663}$ in *E. coli* under the control of either an inducible P_{BAD} promoter (8) or the 182-nucleotide consensus promoter region ($P_{OXA-10}{}^c$) (Table 1). MICs of meropenem against these two strains were determined by the broth microdilution method, as described in the Clinical and Laboratory Standards Institute guidelines (9). The MICs were measured in triplicates in Mueller-Hinton broth (Sigma), with a final inoculum size of 5×10^5 CFU/ml. MIC tests showed that the susceptibility of *E. coli* strains expressing $bla_{OXA-663}$ driven by either $P_{OXA-10}{}^c$ or P_{BAD} in the presence of 2% arabinose does not differ from that of the host *E. coli* strain (Table 2), suggesting that other factors or multiple genes are involved in the resistance mechanism.

To identify the additional factors involved in the resistance mechanism to carbapenems in BIDMC35, we performed RNA sequencing (RNA-seq) of BIDMC35 and two other susceptible but closely related isolates, *K. pneumoniae* UCI7 and UCI64 (4) (Table 2), all belonging to the same multilocus sequence type (ST17). Bacteria were inoculated in Mueller-Hinton broth and at an optical density at 600 nm (OD_{600}) of ~0.1 (time zero), and meropenem was added to individual cultures to a final concentration of 0, 2, 32, or 64 μg/ml. The cells were harvested at 0, 30, 60, and 120 min. RNA-seq libraries were prepared by using an RNA tag-seq protocol (10), and the data were analyzed by using BWA (11) for alignment and DESeq2 (12) for differential expression. RNA-seq results showed that $bla_{OXA-663}$ is constitutively and very highly expressed, regardless of

TABLE 2 Strains and their MICs of meropenem

Strain	MIC of meropenem (μg/ml)	Strain description	Reference or source
BIDMC35	32	Clinical *K. pneumoniae* isolate harboring $bla_{OXA-663}$, ST17	4
MGH71	4	Clinical *K. pneumoniae* isolate harboring bla_{OXA-10} and bla_{KPC-2}, ST258	4
MGH65	8	Clinical *K. pneumoniae* isolate harboring bla_{OXA-10} and $bla_{CTX-M-15}$, ST15	4
UCI64	<0.25	Clinical *K. pneumoniae* isolate, susceptible to carbapenems, ST17	4
UCI7	<0.25	Clinical *K. pneumoniae* isolate, susceptible to Carbapenems, ST17	4
10beta	<0.25	Wild-type *E. coli*	NEB
10beta_P_{BAD}:$bla_{OXA-663}$	<0.25[a]	$bla_{OXA-663}$ expressed in *E. coli* 10beta driven by P_{BAD} promoter	This study
10beta_P_{OXA-10}^{c}:$bla_{OXA-663}$	<0.25	$bla_{OXA-663}$ expressed in *E. coli* 10beta driven by upstream 182-bp consensus region of $bla_{OXA-6\ 63}$ and bla_{OXA-10} (P_{OXA-10}^{c})	This study
BIDMC35 Δ$bla_{OXA-663}$	0.5	Mutant that lost the IncA/C2 plasmid containing $bla_{OXA-663}$	This study
BIDMC35 Δ$bla_{OXA-663}$_P_{BAD}:$bla_{OXA-663}$	16[a]	BIDMC35 Δ$bla_{OXA-663}$ expressing $bla_{OXA-663}$ driven by P_{BAD} promoter	This study
BIDMC35 Δ$bla_{OXA-663}$_P_{BAD}:bla_{OXA-10}	16[a]	BIDMC35 Δ$bla_{OXA-663}$ expressing bla_{OXA-10} driven by P_{BAD} promoter	This study
BIDMC35 *ompK36*$^{+}$	<0.25	BIDMC35 expressing *ompK36* amplified from UCI64	This study

[a]MIC was obtained in Mueller-Hinton broth supplemented with 2% arabinose.

meropenem treatment. In fact, $bla_{OXA-663}$ was always among the top 10 abundant mRNA transcripts in BIDMC35. In contrast, bla_{SHV-1} was expressed at the same level as the other two susceptible strains UCI64 and UCI7. To confirm the RNA-seq results of $bla_{OXA-663}$, we performed reverse transcriptase quantitative PCR (RT-qPCR) for BIDMC35 and MGH71 with or without meropenem treatment (5 μg/ml). The expression level of $bla_{OXA-663}$ was approximately 1,000 times higher in BIDMC35 than the expression level of bla_{OXA-10} in MGH71 (Fig. 1A). Altogether, these results suggested that the expression level of $bla_{OXA-663}$ in BIDMC35 is much higher than the expression level of bla_{OXA-10} in MGH71, indicating that $P_{OXA-663}$ is a stronger promoter than P_{OXA-10}^{c}. This result may explain the inability to obtain transformants expressing $bla_{OXA-663}$ with $P_{OXA-663}$, as the

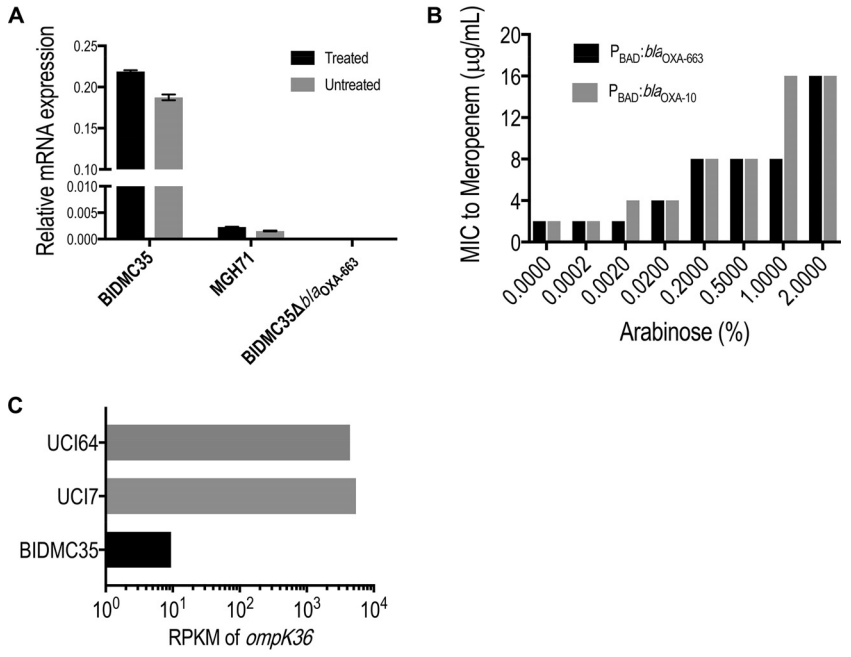

FIG 1 (A) Relative expression levels of $bla_{OXA-663}$ in BIDMC35 and BIDMC35 Δ$bla_{OXA-663}$ and of bla_{OXA-10} in MGH71. RNA was prepared from treated (meropenem at 5 μg/ml) or untreated cultures. The quantification cycle (*Cq*) value was normalized to *Cq* values of 16S rRNA from each sample to calculate the relative expression. (B) MICs of meropenem against BIDMC35 Δ$bla_{OXA-663}$ expressing $bla_{OXA-663}$ or bla_{OXA-10} under the control of the P_{BAD} promoter and with different concentrations of arabinose. (C) RPKM (reads per kilobase of transcript per million mapped reads) of *ompK36* in BIDMC35 and two other susceptible strains, UCI7 and UCI64, (time zero, no meropenem) from the RNA-seq experiment. Data from the other time points and treatment are not shown because they all have similar patterns.

TABLE 3 MICs of selected β-lactam antibiotics against BIDMC35 and relevant strains

Strains	MIC (μg/ml)					
	Meropenem	Imipenem	Ertapenem	Doripenem	Meropenem plus avibactam[a]	Cefotaxime
BIDMC35	32	32	>64	32	32	64
BIDMC35 Δ$bla_{OXA-663}$	0.5	0.5	1	0.25	0.5	1
BIDMC35 Δ$bla_{OXA-663}$–P_{BAD}:$bla_{OXA-663}$	16[b]	16[b]	>64[b]	16[b]	16[b]	64[b]
BIDMC35 Δ$bla_{OXA-663}$–P_{BAD}:bla_{OXA-10}	16[b]	16[b]	>64[b]	16[b]	16[b]	64[b]
BIDMC35 *ompK36*$^{+}$	<0.25	0.5	0.5	0.25	<0.25	16

[a]The concentration of avibactam was fixed at 4 μg/ml, and the values shown are MICs of meropenem.
[b]MIC was obtained in Mueller-Hinton broth supplemented with 2% arabinose.

levels expressed from the multicopy pSMART-LCKan may be toxic to *E. coli*, while the levels expressed with P_{OXA-10}[c] from the same vector may be tolerated.

Given these observations, we hypothesized that the high-level expression of $bla_{OXA-663}$ may be the key to the high-level-carbapenem resistance in BIDMC35. To test this hypothesis, we isolated a clone from BIDMC35 that had lost the IncA/C2 plasmid harboring $bla_{OXA-663}$ (BIDMC35 Δ$bla_{OXA-663}$). The loss of the plasmid and $bla_{OXA-663}$ was confirmed by PCR and RT-qPCR (Fig. 1A). The loss of the IncA/C2 plasmid resulted in a MIC shift of BIDMC35 Δ$bla_{OXA-663}$ into the susceptible range, indicating that a gene(s) on this plasmid is necessary for carbapenem resistance; this result indicated that the chromosomal bla_{SHV-1} does not play a role in carbapenem resistance and further suggested that $bla_{OXA-663}$ might be the relevant gene (Tables 2 and 3). To confirm that it is $bla_{OXA-663}$ rather than the entire plasmid or any other genes on the plasmid that confer resistance to carbapenems, we reintroduced $bla_{OXA-663}$ driven by the inducible P_{BAD} promoter (8) into BIDMC35 Δ$bla_{OXA-663}$ (Table 1). With increasing concentrations of arabinose, carbapenem resistance was restored with MICs correlating with the level of induction of $bla_{OXA-663}$ expression (Fig. 1B). Of note, when we introduced bla_{OXA-10} into BIDMC35 Δ$bla_{OXA-663}$ using the same P_{BAD} plasmid (Table 1), we obtained essentially the same resistance levels as obtained with $bla_{OXA-663}$ (Fig. 1B and Table 3), suggesting that the T16K mutation in $bla_{OXA-663}$ is not critical to the resistance mechanism. This is not surprising, since this mutation is located in signal sequence of OXA-10 (residues 1 to 19) (5). Altogether, these results confirmed that $bla_{OXA-663}$ plays a role in conferring the resistance to carbapenems in BIDMC35 and that its high-level expression is critical to this phenotype. However, in contrast to BIDMC35, the fact that $bla_{OXA-663}$ expression in *E. coli*, even in the presence of high concentrations of arabinose, does not confer carbapenem resistance suggested another factor in BIDMC35 plays a role in carbapenem resistance that distinguishes it from *E. coli*.

Returning to the RNA-seq data for BIDMC35, we noted that in addition to the high expression level of $bla_{OXA-663}$, one of the major porin genes, *ompK36*, was significantly downregulated. Comparing to the other two ST17 susceptible isolates, UCI7 and UCI64, the *ompK36* transcription level (indicated by reads per kilobase per million mapped reads [RPKM]) was approximately 500 times lower (Fig. 1C), indicating that BIDMC35 may be an OmpK36-deficient strain. We examined the DNA sequences surrounding *ompK36* and found that a transposon was inserted at nucleotide position 123 of *ompK36*, disrupting *ompK36*. To test if porin loss is another factor that contributes to the resistance, we episomally introduced an intact *ompK36* gene, including the promoter region of *ompK36* from the susceptible UCI64 (Table 1), into BIDMC35. Restoring a functional OmpK36 porin restored susceptibility to BIDMC35 (Table 3). Of note, since OmpK36 is a major porin for *Klebsiella* species by which they acquire nutrients, we measured the relative growth rates of BIDMC35 after $bla_{OXA-663}$ loss or porin complementation and found no differences (data not shown), suggesting that BIDMC35 may have adapted to compensate for any fitness costs of the resistance mechanisms (13). Altogether, these data show that OmpK36 deficiency is another essential factor that contributes to carbapenem resistance in BIDMC35 and that OmpK36 deficiency and high-level expression of $bla_{OXA-663}$ together result in carbapenem resistance.

These results demonstrate that high-level expression of $bla_{OXA-663}$ and bla_{OXA-10}, regardless of what is traditionally considered their spectrum of activity, can confer carbapenem resistance when expressed at sufficiently high levels in the OmpK36 deficiency background. This work aligns well with an *in vitro* study showing that almost all class D β-lactamases are capable of hydrolyzing carbapenems at low rates (6), and now with confirmation that even β-lactamases with weak carbapenemase activity can result in clinically significant high-level resistance. Of note, the prior *in vitro* demonstration of OXA-10 ability to confer carbapenem resistance was in *A. baumannii*, a Gram-negative pathogen that is notorious for its poor outer membrane permeability (14). Here, we show that the $bla_{OXA-663}$ and bla_{OXA-10} can confer resistance in a *K. pneumoniae* clinical isolate, with the additional requirement of a porin mutation as an alternative solution to impairing carbapenem periplasmic accumulation. Resistance should thus be considered the combinatorial result of several factors, including β-lactamase enzymatic activity, the expression level of the β-lactamase, and carbapenem periplasmic accumulation. Additionally, the impact of a missense mutation in a gene required for resistance, in this case bla_{OXA-10}, on its gene function and thus on a strain's resistance levels cannot be known in the absence of more in-depth investigation, thereby further complicating resistance predictions. In this era of increasing antibiotic resistance and drive for more rapid diagnostics with antimicrobial susceptibility testing, this complexity highlights the challenges and limitations of identifying clinical carbapenem resistance solely on the basis of genotypic identification of β-lactamases with presumed carbapenemase activity.

ACKNOWLEDGMENTS

We thank Jonathan Livny, Nirmalya Bandyopadhyay, Roby Bhattacharyya, Ashlee Earl, Gustavo Cerqueira, and Alejandro Pironti for their helpful discussions. We thank James Gomez for his comments and suggestions on the manuscript.

This study was supported by NIH grant 1R01AI117043-04 (D.T.H.).

We declare that we have no conflicts of interest.

REFERENCES

1. Bradford PA. 2001. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev 14:933–951. https://doi.org/10.1128/CMR.14.4.933-951.2001.
2. Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA. 2011. Carbapenems: past, present, and future. Antimicrob Agents Chemother 55:4943–4960. https://doi.org/10.1128/AAC.00296-11.
3. Meletis G. 2016. Carbapenem resistance: overview of the problem and future perspectives. Ther Adv Infect Dis 3:15–21. https://doi.org/10.1177/2049936115621709.
4. Cerqueira GC, Earl AM, Ernst CM, Grad YH, Dekker JP, Feldgarden M, Chapman SB, Reis-Cunha JL, Shea TP, Young S, Zeng Q, Delaney ML, Kim D, Peterson EM, O'Brien TF, Ferraro MJ, Hooper DC, Huang SS, Kirby JE, Onderdonk AB, Birren BW, Hung DT, Cosimi LA, Wortman JR, Murphy CI, Hanage WP. 2017. Multi-institute analysis of carbapenem resistance reveals remarkable diversity, unexplained mechanisms, and limited clonal outbreaks. Proc Natl Acad Sci U S A 114:1135–1140. https://doi.org/10.1073/pnas.1616248114.
5. Paetzel M, Danel F, de Castro L, Mosimann SC, Page MG, Strynadka NC. 2000. Crystal structure of the class D beta-lactamase OXA-10. Nat Struct Biol 7:918–925. https://doi.org/10.1038/79688.
6. Antunes NT, Lamoureaux TL, Toth M, Stewart NK, Frase H, Vakulenko SB. 2014. Class D beta-lactamases: are they all carbapenemases? Antimicrob Agents Chemother 58:2119–2125. https://doi.org/10.1128/AAC.02522-13.
7. Kim MJ, Bae IK, Jeong SH, Kim SH, Song JH, Choi JY, Yoon SS, Thamlikitkul V, Hsueh PR, Yasin RM, Lalitha MK, Lee K. 2013. Dissemination of metallo-beta-lactamase-producing *Pseudomonas aeruginosa* of sequence type 235 in Asian countries. J Antimicrob Chemother 68:2820–2824. https://doi.org/10.1093/jac/dkt269.
8. Guzman LM, Belin D, Carson MJ, Beckwith J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol 177:4121–4130. https://doi.org/10.1128/jb.177.14.4121-4130.1995.
9. Clinical and Laboratory Standards Institute. 2015. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—10th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
10. Shishkin AA, Giannoukos G, Kucukural A, Ciulla D, Busby M, Surka C, Chen J, Bhattacharyya RP, Rudy RF, Patel MM, Novod N, Hung DT, Gnirke A, Garber M, Guttman M, Livny J. 2015. Simultaneous generation of many RNA-seq libraries in a single reaction. Nat Methods 12:323–325. https://doi.org/10.1038/nmeth.3313.
11. Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754–1760. https://doi.org/10.1093/bioinformatics/btp324.
12. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550. https://doi.org/10.1186/s13059-014-0550-8.
13. Knopp M, Andersson DI. 2015. Amelioration of the fitness costs of antibiotic resistance due to reduced outer membrane permeability by upregulation of alternative porins. Mol Biol Evol 32:3252–3263. https://doi.org/10.1093/molbev/msv195.
14. Moran-Barrio J, Cameranesi MM, Relling V, Limansky AS, Brambilla L, Viale AM. 2017. The *Acinetobacter* outer membrane contains multiple specific channels for carbapenem beta-lactams as revealed by kinetic characterization analyses of imipenem permeation into *Acinetobacter baylyi* cells. Antimicrob Agents Chemother 61:e01737-16. https://doi.org/10.1128/AAC.01737-16.
15. Karunakaran R, Mauchline TH, Hosie AH, Poole PS. 2005. A family of promoter probe vectors incorporating autofluorescent and chromogenic reporter proteins for studying gene expression in Gram-negative bacteria. Microbiology 151:3249–3256. https://doi.org/10.1099/mic.0.28311-0.