Characterization of Proteobacterial Plasmid Integron-Encoded qac Efflux Pump Sequence Diversity and Quaternary Ammonium Compound Antiseptic Selection in Escherichia coli Grown Planktonically and as Biofilms

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ABSTRACT Qac efflux pumps from proteobacterial multidrug-resistant plasmids are integron encoded and confer resistance to quaternary ammonium compound (QAC) antiseptics; however, many are uncharacterized and misannotated. A survey of >2,000 plasmid-carried qac genes identified 37 unique qac sequences that correspond to one of five representative motifs: QacE, QacEΔ1, QacF/L, QacH/I, and QacG. Antimicrobial susceptibility testing of each cloned qac member in Escherichia coli highlighted distinctive antiseptic susceptibility patterns that were most prominent when cells grew as biofilms.

KEYWORDS small multidrug resistance, SMR, efflux pump, disinfectant, antiseptic, quaternary ammonium compound, QAC, qacE, integrons, multidrug-resistant plasmid, multidrug resistance, plasmid-mediated resistance, qacEΔ1, small multidrug resistance proteins

Small multidrug resistance (SMR) family efflux pump genes, annotated as “qac,” are transmitted by proteobacterial integrons carried by multidrug-resistant plasmids (1, 2), making them distinct from other chromosomally inherited SMR members such as Gdx (SugE) and the archetypical member EmrE (3). SMR efflux pump proteins are small (100 to 120 amino acid residues), integral plasma membrane-spanning proteins that act as drug-H+ antiporters. These proteins are composed of only 4 transmembrane α-helices, which multimerize into functional homodimers (1, 4). Qac efflux pumps are named for their ability to confer resistance to quaternary ammonium compound (QAC) antiseptics and have become important genetic biomarkers to predict class 1 integron presence, bacterial antiseptic tolerance, and QAC environmental pollution (5, 6). In proteobacteria, there are numerous integron-associated qac members, annotated as qacE, qacEΔ1, qacF, qacG, qacH, qacL, and qacI in various sequence databases (2, 7, 8); however, only qacE, qacEΔ1, and qacF have been cloned and characterized in Escherichia coli to determine their antimicrobial susceptibility (9–13). qacEΔ1 is the most frequently detected member, because it forms part of the 3’ conserved class 1 integron region. QacEΔ1 is identical to QacE (WP_000679427.1) until its 95th residue, where it has an in-frame insertion element that extends its 4th transmembrane helix by 16 amino acids at the C terminus (Fig. 1), resulting in QacEΔ1 inactivation and reduced ethidium bromide (ET) efflux activity compared to QacE (9). QacE and QacF are predicted to expel a wide range of QACs based on antimicrobial susceptibility testing (AST) (10–13), but the antimicrobial selectivity conferred by other annotated qac sequences (qacG/H/I/L) is inferred from their homology to SMR members. This has resulted in many misannotated qac members and a lack of naming consensus, as well as little comparison of AST methods that involve planktonic, colony, or biofilm growth conditions, which are the main aims of this study.
helices distinguishing QacE from QacE
ment of the 37 unique Qac sequences revealed 5 sequence motifs in all 4 transmembrane
protein sequences for
QacE motif
QacF/L motif
QacH/I motif
QacG motif
TMH regions NH₃

FIG 1 A multiple-protein sequence alignment of the 37 unique Qac sequences identified from this study and a summary of their amino acid sequence
motifs. Amino acids are designated by single-letter abbreviations and color coded based on the Taylor scheme in Jalview software v.2.10.5 (18). The
consensus motif below the main alignment shows each Qac motif. Colored amino acids in each motif indicate unique positions differentiating QacF/L,
QacH/I, QacG, and QacEΔ1. Boldface residues in the consensus and Qac motifs indicate conserved motif positions in the small multidrug protein subclass
of the SMR family. The cylinder diagram at the bottom of the alignment shows the start and ends of the four transmembrane α-helices (TMH) separated
by loops (L1 and L2) and turns (T1) of the known SMR protein secondary structure.

To characterize proteobacterial plasmid qac sequence diversity and homology, we collected 2,953 qac sequences encoded by proteobacterial plasmids deposited in the
GenBank, INTEGRALL (14), UniProt (https://www.uniprot.org), and Comprehensive Antimicrobial Resistance Database (CARD) (15) databases. Plasmid sequences were retrieved using QacE (WP_000679427.1) as a query sequence by tBLASTn (16) analysis. After performing a multiple-sequence alignment of translated Qac sequences with the online server Clustal Omega (17) in Jalview (18), we identified a total of 37 unique Qac protein sequences for final analysis, all with highly variable and inconsistent annotations (Fig. 1; see Tables S1 and S2 in the supplemental material). To accurately classify each Qac sequence, we performed a maximum likelihood phylogenetic analysis using PhyML v.3.0 (19), with archetypical SMR members EmrE (NP_415057.1) and Gdx/SugE (NP_418572.4) as SMR family comparators (Fig. 2). This analysis confirmed that all Qac members were closely related to the SMR family member EmrE, in agreement with previous findings (3, 20). It also revealed that Qac sequences grouped into one of three distinct clades: Qac annotated as (i) QacF/L/H/I, (ii) QacG or QacE, and (iii) QacE and QacEΔ1 (Fig. 2). The alignment of the 37 unique Qac sequences revealed 5 sequence motifs in all 4 transmembrane helices distinguishing QacE from QacEΔ1 and from QacG. The alignment also identified that QacF/L annotated sequences as well as QacH/I were in fact identical to each other (98 to 100% identity) (Fig. 1). Amino acid variations in each Qac motif occurred most often (61 to 82% frequency) at unconserved residue positions in the previously published SMR
FIG 2 The phylogenetic relatedness and taxonomic distribution of the 37 unique Qac sequences identified in this study. Shown is the maximum likelihood dendrogram of the 37 unique Qac protein sequences translated from qac sequences identified in the survey of 2,953 proteobacterial plasmids. Branch node confidence values were determined by performing 1,000 bootstrap replicates using an approximate likelihood ratio testing method. Sequences of archetypical E. coli SMR members EmrE (NP_415057) and Gdx/SugE (NP_418572.4) are highlighted in bold. Other sequences shown in bold indicate qac genes that were selected for AST based on their detection frequency in various plasmids. The taxonomic origins (according to proteobacterial order) of each Qac sequence identified from various plasmids is shown as a bar chart on the right-hand panel of the dendrogram. Numbered bars in this panel indicate the total number of qac sequences identified from plasmids isolated in Enterobacterales (purple), Pseudomonadales (dark blue), Alteromonadales (light blue), Vibrionales (green), and Burkholderiales (red) species, where gray bars indicate indeterminate species.
motif (3, 4) (Fig. 1). Our attempts to identify a Qac sequence progenitor from bacterial genomes using tBLASTn were unsuccessful, as many qac genes are also transmitted on chromosomally integrated integrons as well as plasmids/prophages. Based on the high pairwise sequence identities between each Qac to either QacE or EmrE (Fig. 1), we propose that qac sequences have likely originated from a single qac progenitor incorporated into an integron that is rapidly diverging over time into these qac variants.

This sequence analysis reconfirmed that qacEΔ1 is the predominant qac representative (2,736 qacEΔ1 genes/2,953 total qac genes [92.6%]) (Fig. 2; see Fig. S1 and Table S1 in the supplemental material), given that qacEΔ1 is part of the 3’ conserved region of most class 1 integrons (9). The remaining qac genes were less frequently detected from plasmids (8% of 2,368 plasmids surveyed), where qacG was the second most predominant member (90/2,953 [3.0%]), followed by qacH (13/2,953 [0.4%]), qacF (18/2,953 [0.6%]), qacE (12/2,953 [0.4%]), qacD (8/2,953 [0.3%]), qacC (5/2,953 [0.2%]), qacA (3/2,953 [0.1%]), and qacB (2/2,953 [0.1%]) (Fig. 2; Fig. S1 and Table S1). The majority of all qac sequences identified were from class 1 integrons (93.0 to 100% of all plasmids), with a few qac genes detected at very low frequency (<4%) from class 2 or 3 integrons (Fig. S1). This indicates that qac genes predominate in class 1 integrons, but caution should be taken when using these genes as genetic markers for class 1 integrons.

To determine the substrate selectivity of the five representative Qac sequence motifs, we gene synthesized and cloned qacE (NP_044260.1), qacEΔ1 (YP_003264406.1), qacF (YP_006961976.1), qacH (L0FU64), and qacG (YP_006965429.1) in the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression vector pMS119EH (21) (Tables 1 and 2).
using the same cloning, plasmid expression, and AST methods described in a recent study of *qdx/sugE* (22). We chemically transformed each plasmid into *E. coli* K-12 BW25113 (wild type) (23), as well as strain KAM32 (24), which lacks a competing dominant efflux pump gene, *acrB*, and an additional efflux pump gene, *mdtK*, improving *qac* substrate selectivity determination by AST. To determine differences in antiseptic resistance that may be attributed to different cell growth physiologies, as noted in our previous study (21), we performed three different AST culturing techniques in Luria-Bertani (LB) medium with 100 μg/ml ampicillin and 0.05 mM IPTG addition to determine the MICs for each cloned vector transformant. We measured planktonic growth using 96-well broth microdilution plating techniques and cell colony growth on agar spot plating as described by Slipski et al. (22). We also determined the minimal biofilm eradication concentration (MBEC) for transformants grown as biofilms using the MBEC device (Innovotech, Inc., Canada) as described in reference 22. All AST involved a library of 13 antimicrobials commonly tested in previous SMR studies (as reviewed in reference 4) (Tables 1 and 2). For AST, we applied a significant difference (within a 2-fold MIC difference or identical) to the vector control pMS119EH, with the exception of the pEmrE transformant (Table 1). BW25113/pEmrE transformant agar colony AST results showed higher QAC resistance (MIC values of ≥4-fold) to ethidium bromide (ET) and acriflavine

| TABLE 2 | Summary of AST MIC and MBEC values determined for *E. coli* KAM32 transformed with various *qac* vectors and grown as planktonic (broth), colony (agar spot), and biofilm (MBEC) cultures for 24 h at 37°C |
| Condition and vector | MIC or MBEC (μg/ml) |
|----------------------|---------------------|
|                      | CPC | CET | BZK | DDAB | CDEB | CTAB | DOM | ET | AC | MV | CHX | ERY | TOB |
| Agar (n = 3)          |     |     |     |      |      |      |     |    |    |    |     |     |     |
| pMS119EH             | 2   | 1   | 1   | 0.5  | 2    | 4    | 2   | 12 | 1  | 80 | 4   | 2   | 4   |
| pEmrE                | 8   | 4   | 2   | 2    | 8    | 8    | 4   | 256 | 8 | 160 | 4   | 2   | 2   |
| pQacE                | 8   | 8   | 4   | 1    | 8    | 8    | 4   | 32 | 4  | 80 | 4   | 2   | 2   |
| pQacEΔ1             | 2   | 4   | 1   | 0.5  | 4    | 4    | 2   | 8  | 4  | 80 | 4   | 2   | 4   |
| pQacF               | 8   | 8   | 4   | 2    | 8    | 8    | 4   | 8  | 4  | 80 | 4   | 2   | 8   |
| pQacG               | 8   | 8   | 4   | 2    | 8    | 4    | 2   | 8  | 1  | 80 | 4   | 2   | 2   |
| pQacH               | 8   | 4   | 4   | 2    | 8    | 8    | 8   | 32 | 4  | 80 | 4   | 2   | 4   |
| Broth (n = 3)         |     |     |     |      |      |      |     |    |    |    |     |     |     |
| pMS119EH             | 1.2 | 2   | 2   | 0.5  | 2    | 1    | 1   | 16 | 8  | 80 | 1   | 1–2 | 2   |
| pEmrE                | 2.4 | 4   | 2   | 1    | 2    | 1    | 1   | 1–2| 64 | 64 | 320 | 1   | 1–2 | 2–4 |
| pQacE                | 1.2–2.4 | 4 | 2   | 1    | 2    | 1   | 1–2 | 64 | 64 | 16–32 | 80 | 1   | 1–2 | 2   |
| pQacEΔ1             | 1.2–2.4 | 2 | 2   | 0.5  | 2    | 1    | 1   | 32 | 8  | 80 | 1   | 1   | 2   |
| pQacF               | 1.2–2.4 | 8 | 4   | 2    | 2    | 2    | 1–2 | 16 | 8  | 80 | 1   | 2   | 2–4 |
| pQacG               | 2.4 | 4   | 4   | 2    | 4    | 2    | 1–2 | 16 | 8  | 160 | 1  | 2   | 2–4 |
| pQacH               | 1.2–2.4 | 4 | 4   | 2    | 4    | 1   | 1–2 | 32 | 16–32 | 160 | 1  | 1–2 | 2–4 |
| MBEC (n = 6)          |     |     |     |      |      |      |     |    |    |    |     |     |     |
| pMS119EH             | 128 | 16  | 32  | 16   | 16   | 16   | 8   | 8  | 64 | 32 | 4,096 | 32 | 64 | 2–4 |
| pEmrE                | 32  | 32  | 16  | 16   | 64   | 8    | 26  | 512| 256| 1,6384 | 64 | 128 | 8–16 |
| pQacE                | 32  | 32  | 16  | 8    | 32–64 | 8   | 16  | 256| 128–256 | 1,6384 | 64 | 128 | 8   |
| pQacEΔ1             | 64  | 64  | 32  | 6    | 16   | 4    | 8   | 64 | 64 | 1,6384 | 64 | 64–128 | 8   |
| pQacF               | 64  | 64  | 32  | 16   | 32–64 | 4   | 32  | 256| 64  | 4,096 | 32 | 64  | 16  |
| pQacG               | 256 | 128 | 32  | 16   | 32–64 | 16  | 8   | 32–64| 16–32| 1,6384 | 64 | 64  | 8   |
| pQacH               | 32  | 16  | 16  | 16   | 16   | 32–64| 16  | 3   | 341| 256  | 4,096| 64  | 128 | 16–32 |

*a*Antimicrobial abbreviations: CPC, cetylpyridinium chloride; CET, cetrimide bromide; BZK, benzalkonium chloride; DDAB, didodecyldimethylammonium bromide; CDEB, cetylmyethylhexadecylammonium bromide; CTAB, cetyltrimethylammonium bromide; DOM, domiphen bromide; ET, ethidium bromide; MV, methyl viologen dichloride; CHX, chlorhexidine; dichloride; AC, acriflavine; ERY, erythromycin; TOB, tobramycin. Boldface numbers indicate ≥4- or ≥4-fold changes in MIC/MBEC AST values compared to pMS119EH transformants under the same AST growth conditions.

*AST involved 10−4 dilutions of plasmid-transformed cultures adjusted to an optical density at 600 nm of 1.0 as the starting inoculum in Luria-Bertani medium with 100 μg/ml ampicillin and 0.05 mM isopropyl β-d-1-thiogalactopyranoside (IPTG).

*Shown are results from 24-h biofilms grown on the MBEC device pegged lid incubated with drug for 24 h in LB broth prior to AST.

*All genes were directionally cloned in the multiple-cloning site of pMS119EH at 5′ XbaI and 3′ HindIII restriction sites.

October 2021 Volume 65 Issue 10 e01069-21 aac.asm.org

Proteobacterial Plasmid-Transmitted qac Efflux Pumps

Antimicrobial Agents and Chemotherapy
and 2), as reported for previous agar plate AST studies of cetyltrimethylammonium bromide [CTAB], and benzalkonium chloride [BZK]) (Tables 1 and 2). KAM32 transformed with pEmrE or pQac vectors (including pQacE/D) expectedly conferring resistance to the fewest substrates (cetrimide bromide [CET] and MV in Table 1). Therefore, in the wild-type efflux pump BW25113 strain, pQacE, pQacF, pQacH, and pQacG transformants grown as biofilms conferred resistance to the broadest range of antimicrobials (6 QACs plus tobramycin [TOB]), with pQacE, -F/L, or -G transformants resistant to 3 to 4 antimicrobials, and pQacEΔ1 unexpectedly conferring resistance to the fewest substrates (cetrimide bromide [CET] and MV in Table 1). These findings show that AST in strains lacking competing efflux pumps helped identify a broader range of QACs selected for by each qac gene when grown planktonically or as colonies. The KAM32 agar AST profile of recognized antimicrobial compounds was unique for each pQac transformant we tested, reflecting their sequence motif differences (Fig. 1). BW25113/pQacH transformant biofilms conferred resistance to the broadest range of antimicrobials (6 QACs plus tobramycin [TOB]), with pQacE, -F/L, or -G transformants resistant to 3 to 4 antimicrobials, and pQacEΔ1 expectedly conferring resistance to the fewest substrates (cetrimide bromide [CET] and MV in Table 1). Therefore, in the wild-type efflux pump BW25113 strain, pQacE, pQacF, pQacH, and pQacG transformants grown as biofilms confer unique antimicrobial resistance profiles to a limited range of QACs compared to pEmrE (Table 1).

To improve substrate selection identification conferred by each representative qac, we repeated AST with KAM32 ΔacrB ΔmdtK/pQac transformants (Table 2). As previously reported (22, 27), KAM32 has slower growth and higher drug susceptibility than BW25113, resulting in lower MIC and MBEC values for all antimicrobials we tested compared to BW25113/pMS119EH (Table 2). Broth and agar spot plate AST results for KAM32 transformed with pEmrE or pQac vectors (including pQacEΔ1) demonstrated a significant increase (>4-fold) in MIC values for one or more QACs compared to pMS119EH (Table 2) or compared to the same AST results from BW25113 transformants (Table 1). These findings show that AST in strains lacking competing efflux pumps helps identify a broader range of QACs selected for by each qac gene when grown planktonically or as colonies. The KAM32 agar AST findings are in agreement with previous qac studies, as we identified increased resistance to similar QAC substrates (ET, cetyltrimethylammonium bromide [CTAB], and benzalkonium chloride [BZK]) (Tables 1 and 2), as reported for previous agar plate AST studies of qacE and qacEΔ1 (9, 12, 13), as well as QacF (10, 11). However, KAM32/pQac transformant biofilm AST unexpectedly identified fewer antimicrobials that significantly increased MBEC values compared to the control vector (Table 2) or compared to BW25113 biofilm results (Table 1). KAM32 transformant biofilm MBEC results identified enhanced antimicrobial susceptibility (≤4-fold reduction in MBEC values) for pQacEΔ1 and pQacF exposed to QACs, CET, BZK, dicocymidemethylammonium bromide (DDAB), and CTAB (Table 2). Enhanced susceptibility was also observed for biofilm BW25113/pEmrE and -pQacE transformants for CTAB (Table 1). This suggests that overexpression of these qac efflux pumps works against the cell under these biofilm growth conditions, making cells more susceptible to the aforementioned QACs. The ability of SMR members to confer enhanced antimicrobial susceptibility in the presence of different antimicrobials has been reported in previous studies (28, 29) and may be due to amino acid variations that switch these pumps from exporters to importers for these particular drugs.

In conclusion, our findings reveal that many proteobacterial plasmid-carried qac genes are misannotated in sequencing databases, and the comprehensive Qac motif comparison herein can improve annotation of qac variants. We observed that qacH/I variants had the broadest antimicrobial recognition profile when grown as biofilms, whereas qacEΔ1 transformants conferred significant QAC resistance to the smallest number of QACs (CET and MV), indicating that even this relatively inactive qac variant can still confer limited QAC resistance. Our analysis also importantly shows that qac efflux pumps are most effective when E. coli cells grow as a biofilm and least effective when cells grow as planktonic cultures, which is concerning when considering that biofilm prevention and eradication strategies frequently rely on the use of QAC.

October 2021 Volume 65 Issue 10 e01069-21 aac.asm.org

Slipski et al. Antimicrobial Agents and Chemotherapy
disinfectants (30). Altogether, this information provides more context to ongoing antimicrobial resistance genetic surveillance studies by providing qac-specific antimicrobial phenotypes to uncharacterized qac genes, clear and improved annotations, and identification of optimal growth physiologies influencing their conferred phenotypes.

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.22 MB.

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REFERENCES

1. Bay DC, Turner RJ. 2016. Small multidrug resistance efflux pumps, p 45–71. In Li X, Elkins CA, Zgurskaya HI (ed), Efflux-mediated antimicrobial resistance in bacteria. Springer, New York, NY.

2. Jaglicz Z, Cervinkova D. 2012. Genetic basis of resistance to quaternary ammonium compounds—the qac genes and their role: a review. Veterinarni Medicina 57:275–281. https://doi.org/10.17221/6013-VETMED.

3. Bay DC, Turner RJ. 2009. Diversity and evolution of the small multidrug resistance protein family. BMC Evol Biol 9:140. https://doi.org/10.1186/1471-2148-9-140.

4. Bay DC, Rommens KL, Turner RJ. 2008. Small multidrug resistance proteins: a multidrug transporter family that continues to grow. Biochim Biophys Acta 1778:1814–1838. https://doi.org/10.1016/j.bbamem.2007.08.015.

5. Szekeres E, Chiriac CM, Baricz A, Szőke-Nagy T, Lung I, Soran ML, Rudi K, Dragos N, Coman C. 2018. Investigating antibiotics, antibiotic resistance genes, and microbial contaminants in groundwater in relation to the proximity of urban areas. Environ Pollut 236:734–744. https://doi.org/10.1016/j.envpol.2018.01.107.

6. Gaze WH, Abdousslam N, Hawksey PM, Wellington EM. 2005. Incidence of class 1 integrons in a quaternary ammonium compound-polluted environment. Antimicrob Agents Chemother 49:1802–1807. https://doi.org/10.1128/AAC.49.5.1802-1807.2005.

7. Gillings MR, Xuejun D, Hardwick SA, Holley MP, Stokes HW. 2009. Gene cassettes encoding resistance to quaternary ammonium compounds: a role in the origin of clinical class 1 integrons? ISME J 3:209–215. https://doi.org/10.1038/ismej.2008.98.

8. Gillings MR, Holley MP, Stokes HW. 2009. Evidence for dynamic exchange of qac gene cassettes between class 1 integrons and other integrons in freshwater biofilms. FEMS Microbiol Lett 296:282–288. https://doi.org/10.1111/j.1574-6968.2009.01646.x.

9. Paulsen IT, Littlejohn TG, Radstrom P, Sundstrom L, Skold O, Swedberg G, Skurray RA. 1993. The 3′ conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. Antimicrob Agents Chemother 37:761–768. https://doi.org/10.1128/AAC.37.4.761.

10. Ploy MC, Courvalin P, Lambert T. 1998. Characterization of in40 of Enterobacter aerogenes BM2688, a class 1 integron with two new gene cassettes, cmlA2 and qacE. Antimicrob Agents Chemother 42:2557–2563. https://doi.org/10.1128/AAC.42.10.2557.

11. Schluter A, Heuer H, Szczepanowski R, Poler SM, Schnieker S, Pühler A, Top EM. 2005. Plasmid pB8 is closely related to the prototype IncP-1beta plasmid R751 but transfers poorly to Escherichia coli and carries a new transposon encoding a small multidrug resistance efflux protein. Plasmid 54:135–148. https://doi.org/10.1016/j.plasmid.2005.03.001.

12. Kazama H, Harashima H, Sasatsu M, Arai T. 1999. Characterization of the antiseptic-resistance gene qacEdelta1 isolated from clinical and environmental isolates of Vibrio parahaemolyticus and Vibrio cholerae non-O1. FEMS Microbiol Lett 174:379–384. https://doi.org/10.1111/j.1574-6968.1999.tb15393.x.

13. Kücken D, Feucht H, Kaulfers P. 2000. Association of qacE and qacEdelta1 with multiple resistance to antibiotics and antiseptics in clinical isolates of Gram-negative bacteria. FEMS Microbiol Lett 183:95–98. https://doi.org/10.1111/1574-6968.2000.tb08939.x.

14. Moura A, Soares M, Pereira C, Leitão N, Henriques I, Correia A. 2009. INTEGRALL: a database and search engine for integrons, integrases and gene cassettes. Bioinformatics 25:1096–1098. https://doi.org/10.1093/bioinformatics/btp105.

15. McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, Bhullar K, Canova MJ, De Pascale G, Eijm L, Kalan L, King AM, Koteva K, Morar M, Mulvey MR, O’Brien JS, Pawlowski AC, Piddock LJV, Spanogiannopoulos P, Sutherland AD, Tang I, Taylor PL, Thaker M, Wang Y, Yan M, Yu T, Wright GD. 2013. The comprehensive antibiotic resistance database. Antimicrob Agents Chemother 57:3348–3357. https://doi.org/10.1128/AAC.00419-13.

16. Gertz EM, Yu YK, Agarwala R, Schaffer AA, Altschul SF. 2006. Composition-based statistics and translated nucleotide searches: improving the TBLASTN module of BLAST. BMC Biol 4:41. https://doi.org/10.1186/1741-7007-4-41.

17. Sievers F, Higgins DG. 2014. Clustal Omega. Curr Protoc Bioinformatics 48:3.13.1–3.13.16. https://doi.org/10.1002/0471250953.bi0313s48.

18. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. 2009. Jalview version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics 25:1189–1191. https://doi.org/10.1093/bioinformatics/btp093.

19. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59:307–321. https://doi.org/10.1093/sysbio/syq010.

20. Kermanni AA, Macdonald CB, Gundeepudri R, Stockbridge RB. 2018. Guanidinium export is the primal function of Smr family transporters. Proc Natl Acad Sci U S A 115:3060–3065. https://doi.org/10.1073/pnas.1719187115.

21. Furste JP, Pansegrau W, Frank R, Blocker H, Schulz P, Bagdasarian M, Lanka E. 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range tac expression vector. Gene 48:119–131. https://doi.org/10.1016/0378-1119(86)90358-6.
22. Slipski CJ, Jamieson TR, Zhanel GG, Bay DC. 2020. Riboswitch-associated guanidinium selective efflux pumps frequently transmitted on proteobacterial plasmids increase Escherichia coli biofilm tolerance to disinfectants. J Bacteriol 202:e00104-20. https://doi.org/10.1128/JB.00104-20.

23. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KAA, Tomita M, Wanner BLL, Mori H. 2006. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2:2006–2008. https://doi.org/10.1038/msb4100050.

24. Chen J, Morita Y, Huda MN, Kuroda T, Mizushima T, Tsuchiya T. 2002. VmrA, a member of a novel class of Na\(^{+}\)-coupled multidrug efflux pumps from Vibrio parahaemolyticus. J Bacteriol 184:572–576. https://doi.org/10.1128/JB.184.2.572-576.2002.

25. Tal N, Schuldiner S. 2009. A coordinated network of transporters with overlapping specificities provides a robust survival strategy. Proc Natl Acad Sci U S A 106:9051–9056. https://doi.org/10.1073/pnas.0902400106.

26. Morimyo M, Hongo E, Hama-Inaba H, Machida I. 1992. Cloning and characterization of the mvrC gene of Escherichia coli K-12 which confers resistance against methyl viologen toxicity. Nucleic Acids Res 20:3159–3165. https://doi.org/10.1093/nar/20.12.3159.

27. Bay DC, Stremick CA, Slipski CJ, Turner RJ. 2017. Secondary multidrug efflux pump mutants alter Escherichia coli biofilm growth in the presence of cationic antimicrobial compounds. Res Microbiol 168:208–221. https://doi.org/10.1016/j.resmic.2016.11.003.

28. Brill S, Falk OS, Schuldiner S. 2012. Transforming a drug/H\(^{+}\) antiporter into a polyamine importer by a single mutation. Proc Natl Acad Sci U S A 109:16894–16899. https://doi.org/10.1073/pnas.1211831109.

29. Son MS, Del Castilho C, Dunclaf KA, Carney D, Weiner JH, Turner RJ. 2003. Mutagenesis of SugE, a small multidrug resistance protein. Biochem Biophys Res Commun 312:914–921. https://doi.org/10.1016/j.bbrc.2003.11.018.

30. Verderosa AD, Totsika M, Fairfull-Smith KE. 2019. Bacterial biofilm eradication agents: a current review. Front Chem 7:824. https://doi.org/10.3389/fchem.2019.00824.