Na\textsuperscript{+}-NQR Confers Aminoglycoside Resistance via the Regulation of L-Alanine Metabolism

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\textbf{ABSTRACT} Sodium-translocating NADH:quinone oxidoreductase (Na\textsuperscript{+}-NQR) functions as a unique redox-driven sodium pump, generating membrane potential, which is related to aminoglycoside antibiotic resistance. However, whether it modulates other metabolisms to confer antibiotic resistance is unknown. The present study showed that loss of \textit{nqrA} or \textit{nqrF} led to differential metabolomes with elevated resistance to aminoglycoside antibiotics. Decreased alanine, aspartate, and glutamate metabolism and depressed abundance of alanine were characterized as the most impacted pathway and crucial biomarker, respectively. Further data showed that higher viability was detected in \textit{ΔnqrA} and \textit{ΔnqrF} mutant strains than their parent strain ATCC 33787 in the presence of gentamicin but recovered by exogenous L-alanine. It proceeds by the following events. The loss of \textit{nqrA} or \textit{nqrF} led to the decrease of membrane potential, ATPase activity, and then ATP and cyclic AMP (cAMP), which reduced the cAMP/CRP (cAMP receptor protein) complex. The reduced cAMP/CRP complex promoted L-alanine catabolism and inhibited L-alanine anabolism, causing reduced levels of alanine. Reduced alanine affected the expression of antipporter families Atp and Mnh genes. Our results suggest a novel mechanism by which the Na\textsuperscript{+}-NQR system regulates antibiotic resistance via L-alanine metabolism in a cAMP/CRP complex-dependent manner.

\textbf{IMPORTANCE} The Na\textsuperscript{+}-NQR complex functions as a unique redox-driven sodium pump, generating membrane potential directly. However, whether it mediates generation of membrane potential indirectly is unknown. The present study shows that the Na\textsuperscript{+}-NQR complex impacts membrane potential through other antipporter families Atp and Mnh. It proceeds by ATP and then cAMP/CRP regulon, which inhibits L-alanine catabolism and promotes L-alanine anabolism. When the Na\textsuperscript{+}-NQR complex is reduced as in antibiotic-resistant bacteria, L-alanine is depressed, which is related to the antibiotic resistance phenotypes. However, exogenous L-alanine reverts the phenotype and promotes antibiotic-mediated killing. These findings suggest a novel mechanism by which the Na\textsuperscript{+}-NQR system regulates antibiotic resistance via L-alanine metabolism in a cAMP/CRP complex-dependent manner.

\textbf{KEYWORDS} Na\textsuperscript{+}-NQR, alanine metabolism, aspartate metabolism, glutamate metabolism, alanine, aminoglycoside antibiotics, cAMP/CRP, antibiotic resistance, metabolomics

Within a relatively short time after the first antibiotics were introduced, bacteria began exhibiting various degrees of resistance (1). Antibiotic resistance is a global challenge that impacts all pharmaceutically used antibiotics (2, 3). In response to
the emergent antibiotic resistance, novel strategies are needed to develop new antibiotics, vaccines, and bacitracin (4–9) and to enhance the effectiveness of existing antibiotics (10–13). Therefore, the understanding of antibiotic resistance mechanisms and identification of drug targets to battle these emerging antibiotic-resistant pathogens deserve priority status.

The sodium-translocating NADH-ubiquinone oxidoreductase (Na⁺-NQR) is found in the electron transport chain of several pathogenic and marine bacteria, including vibrios, functioning in a unique redox-driven sodium pump (14). It is a multisubunit (NqrA-F) membrane-embedded NADH dehydrogenase that oxidizes NADH and reduces quinone to quinol. It has been proposed that a scheme of electron transfer in Na⁺-NQR is initiated by NADH oxidation on subunit NqrF and leads to quinol formation on subunit NqrA. Moreover, recent reports have indicated that Na⁺-NQR plays a role in bacterial metabolism, motility, and resistance to antibiotics and osmotic stress (15–17). The lack of Na⁺-NQR harms cholera toxin levels via affecting either translation or secretion (18). The recent breakthrough in structural studies on Na⁺-NQR from Vibrio cholerae creates a perspective for the systematic design of inhibitors for this unique enzyme as a novel target for antibiotics (19). Therefore, further understanding of the role of Na⁺-NQR in antibiotic resistance is especially important to regulate the enzyme for combating these antibiotic-resistant pathogens.

Recent reports have indicated that bacterial metabolomes contribute to susceptibility and resistance to antibiotics and serum-mediated killing (10, 20–24). Therefore, it is interesting to investigate whether Na⁺-NQR regulates antibiotic resistance through metabolic modulation. The present study demonstrates that loss of nqrA or nqrF leads to the decreased alanine, aspartate, and glutamate metabolism and the depressed abundance of alanine, which contributes to antibiotic resistance. The decrease of alanine is attributed to the regulation of nqrA and nqrF to L-alanine metabolism in a cyclic AMP (cAMP)/cAMP receptor protein (CRP)-dependent manner. Furthermore, L-alanine abundance is related to the membrane potential and intracellular concentration of gentamicin via regulating other antiporter families Atp and Mnh. The results are described below.

RESULTS

Resistance of ΔnqrA and ΔnqrF mutant strains to aminoglycoside antibiotics.

To understand the role of nqrA or nqrF in resistance to aminoglycosides, genetically modified mutants with nqrA or nqrF deleted were constructed (see Fig. S1 in the supplemental material). We first detected the growth curves of Vibrio alginolyticus ATCC 33787 and ΔnqrA and ΔnqrF mutant strains (ΔnqrA and ΔnqrF, respectively) and found that the loss of nqrA or nqrF leads to reduced growth in the exponential phase (Fig. 1A). Then, MICs of V. alginolyticus ATCC 33787, ΔnqrA, and ΔnqrF to three types of aminoglycoside antibiotics (amikacin, gentamicin, and kanamycin) were measured. The loss of nqrA and nqrF led to a fourfold elevation of MICs in medium with the three types of antibiotics except for a twofold elevation of the MIC to amikacin in ΔnqrA (Fig. 1B). These results indicate that the absence of nqrA or nqrF increases bacterial resistance to these antibiotics.
Metabolomic profiling of ΔnqrA and ΔnqrF. Reports have indicated that bacterial metabolomes contribute to antibiotic efficacy (10, 11, 23). To explore whether the loss of nqrA or nqrF affects metabolic profiles, gas chromatography-mass spectrometry (GC-MS)-based metabolomics was used to detect metabolomes of ΔnqrA and ΔnqrF. Five biological replicates with two technical repeats in each group were performed, yielding a total of 30 data sets (Fig. S2A). This led to identification of 80 metabolites from each sample. The high reproducibility of the identification in the discovery phase is shown in Fig. S2B. The biological categories of the identified metabolites were searched for in the Kyoto Encyclopedia of Genes and Genomes (KEGG). The categories showed that 18.75% (16), 32.50% (26), 27.50% (22), and 16.25% (13) of metabolites belong to carbohydrates, amino acids, lipids, and nucleotides, respectively (Fig. S2C).

Compared with the metabolome of *V. alginolyticus* ATCC 33787, 42 (52.5%) and 48 (60.0%) metabolites showed differential abundances ($P < 0.05$) in ΔnqrA and ΔnqrF, respectively (Fig. 2A). Z-values based on the control group were calculated, showing that it spanned from −8.82 to 22.48 in ΔnqrA and from −10.82 to 92.49 in ΔnqrF (Fig. 2B). Specifically, 23 metabolites were decreased and 19 metabolites were increased in ΔnqrA, and 23 metabolites were decreased and 25 metabolites were increased in ΔnqrF. We further examined the metabolic categories of these differential metabolites. They showed differential percentages with a ranking of amino acids > lipids > carbohydrates > nucleotides in both ΔnqrA and ΔnqrF (Fig. 2C). The numbers of up- and downregulated metabolites in these categories are shown in Fig. 2D. These results indicate that the loss of nqrA or nqrF affects bacterial metabolism.

Enrichment of metabolic pathways involved in ΔnqrF and ΔnqrA. Furthermore, metabolic pathways of this differential abundance of metabolites were analyzed. First, a comparative analysis of the differential metabolites was performed between ΔnqrF and ΔnqrA. Among the 42 and 48 differential abundance of metabolites, respectively, in ΔnqrA and ΔnqrF, 37 metabolites overlapped, 18 were decreased, 16 were increased. Three metabolites, guanosine, propionic acid, and valine, were differentially abundant between ΔnqrA and ΔnqrF. The others were ΔnqrA and ΔnqrF specific, including 2 increased metabolites and 3 decreased metabolites in ΔnqrA and 7 increased metabolites and 4 decreased metabolites in ΔnqrF (Fig. 3A). These results indicate that most of the metabolites with differential abundance overlapped in the two mutants since they together are a part of the sodium-translocating NADH:quinone oxidoreductase.

Eight pathways were enriched by the shared differential metabolites between ΔnqrA and ΔnqrF. The most impactful pathway is alanine, aspartate, and glutamate metabolism, followed by glycine, serine, and threonine metabolism, arginine and proline metabolism, glutathione metabolism, aminoacyl-tRNA biosynthesis, nitrogen metabolism, cyanoamino acid metabolism, and benzoate degradation via coenzyme A (CoA) ligation (Fig. 3B). Except for benzoate degradation via CoA ligation, in which the two differential metabolites detected were increased in abundance, the other pathways showed most or half of the differential metabolites were decreased in abundance. Among these metabolites in these pathways, all showed the same trend of changes in abundance between ΔnqrA and ΔnqrF except for valine, which was decreased in ΔnqrA and increased in ΔnqrF (Fig. 3C). These results indicate that the loss of either nqrA or nqrF leads to similar changes in the enriched metabolic pathways.

Identification of crucial biomarkers using multivariate data analysis. To identify crucial biomarkers representing differential metabolomes due to the absence of nqrA or nqrF, orthogonal partial least-squares discriminant analysis (OPLS-DA) was applied for the recognition of the sample patterns, followed by ranking the altered abundance of metabolites in loading. ΔnqrA and ΔnqrF were separated from the control group clearly by predictive component 1, and the two mutants were separated by predictive component 2 (Fig. 3D). Discriminating variables were present with S-plot when cutoff values were set at greater or equal to 0.05 and 0.5 for absolute value of covariance ($P$) and correlation ($r$), respectively. Ornithine, alanine, proline, glutamic acid, aspartic acid, penicillioic acid, itaconic acid, pipecolinic acid, ethanolamine, adenine, do-
decanoic acid, tyrosine, valine, lysine, and glycine are displayed as the metabolites that have the first 15 largest correlations and covariances in predictive component 1 between the two mutants and control. Phenethylamine, propionic acid, and valine are identified as the biomarkers that have the first three largest correlations and covariances in predictive component 2 between ΔnqrA and ΔnqrF (Fig. 3E). Among the first 15 largest correlations and covariances in predictive component 1, 5 metabolites...
lysine, tyrosine, dodecanoic acid, glycine and valine) were increased and 10 metabolites (itaconic acid, alanine, pipecolinic acid, pentanedioic acid, glutamic acid, ornithine, aspartic acid, proline, adenosine, and ethanolamine) were decreased (Fig. 3F). Out of these metabolites, alanine, aspartic acid, and glutamic acid belong to alanine, aspartate, and glutamate metabolism, the biggest impact pathway. Among the three, alanine has

FIG 3

Shared pathways, metabolites, and multivariate data analysis between ΔnqrA and ΔnqrF strains. (A) Venn diagram showing the overlapping and unique differential metabolites between ΔnqrA and ΔnqrF strains. Decreased and increased metabolites are indicated with down and up arrows, respectively. (B) Enriched pathways by the 37 overlapping metabolites between the two test groups. (C) Integrative analysis of metabolites in significantly enriched pathways. Yellow and blue indicate an increase and decrease of metabolites, respectively. The number shows the relative value of differential abundance of metabolites. VA, V. alginolyticus ATCC 33787. (D) PCA analysis of control, ΔnqrA, and ΔnqrF. Each dot represents the technical replicate of samples in the plot. (E) S-plot generated from OPLS-DA. Predictive component p[1] and correlation p(corr)[1] differentiate ΔnqrA and ΔnqrF from VA. Predictive component p[2] and correlation p(corr)[2] separate ΔnqrF from ΔnqrA. The triangle represents metabolites in which candidate biomarkers are marked. (F) Scatter diagram of 15 biomarkers. *, P < 0.05; **, P < 0.01.
the most absolute value of covariance $p$ and the most difference in abundance between ATCC 33787 and ΔnqrA or ΔnqrF. Therefore, alanine is identified as the most crucial biomarker.

**Exogenous L-alanine promotes gentamicin-mediated killing.** Since the complement of depressed amino acids in the metabolome potentiates antibiotics to kill antibiotic-resistant *Edwardsiella tarda*, *Escherichia coli*, and *V. alginolyticus* (10, 20, 23), we supposed that exogenous alanine could cope with the resistance due to the loss of nqrA or nqrF. To demonstrate this, we first showed that higher survival was detected in ΔnqrA and ΔnqrF than in their parent strain *V. alginolyticus* ATCC 33787 in a gentamicin dose-dependent manner (Fig. 4A). Then, we performed the L-alanine-enabled killing of *V. alginolyticus* ATCC 33787, ΔnqrA, and ΔnqrF, showing higher survival of ΔnqrA or ΔnqrF than of ATCC 33787 in medium with 0.3 to 1.2 mM L-alanine, but survival returned to normal in medium with 5 to 20 mM L-alanine (Fig. 4B; Fig. S3). A similar
effect but higher viability was detected in the replacement of alanine with aspartate or glutamate in the same metabolic pathway (Fig. 4C and D). Moreover, the L-alanine-enabled killing was determined in a gentamicin- and time-dependent manner. A significant difference was detected between control and ΔnqrA or ΔnqrF but not when 10 mM L-alanine was supplemented (Fig. 4E and F). The amount of alanine was quantified and compared to cells cultured in the absence of the antibiotic and alanine. Lower alanine was detected in ΔnqrA and ΔnqrF than ATCC 33787 in control, only the gentamicin group (except for ΔnqrA) and only alanine group, while higher alanine was measured in ΔnqrA and ΔnqrF than ATCC 33787 in the synergistic use of gentamicin and alanine group (Fig. 4G). However, 10 mM L-alanine did not change the MIC of ΔnqrA or ΔnqrF (data not shown), which may be related to the fact that MIC was measured in LB medium, which contains complex nutrients for bacterial use. We also showed that decreased abundance of NqrA and NqrF exists in clinically isolated strains VA2 and VA3 (16 and 32 MIC to gentamicin, respectively) (Fig. 4H). In E. coli, nuoC, nuoF, and nuoG encoding the proton-pumping NADH-ubiquinone oxidoreductase play roles similar to the roles of nqrA and nqrF NQR complex (the sodium-translocating NADH:quinone oxidoreductase) in the respiratory chain. nuoC, nuoF, and nuoG-dependent alanine depression and aminoglycoside resistance were characterized (Fig. 4I to K). Consistently, exogenous L-alanine potentiated gentamicin to kill both clinically isolated Vibrio species and E. coli strains (Fig. S4). These results indicate that exogenous L-alanine not only promotes the sensitivity of Vibrio species and E. coli to gentamicin but also reverts the gentamicin resistance due to the loss of nqrA or nqrF.

L-Alanine restores levels of membrane potential, intracellular gentamicin, and activity of enzymes in the pyruvate cycle due to the loss of nqrA or nqrF. Reports have indicated that aminoglycoside antibiotic uptake is dependent on the proton motive force (PMF) (25), which motivated us to explore whether exogenous L-alanine restores the level of membrane potential due to the absence of nqrA or nqrF. To test this, the membrane potential was detected in ATCC 33787, ΔnqrA, and ΔnqrF. The loss of nqrA or ΔnqrF led to the decrease in membrane potential, but it recovered in the presence of 10 mM L-alanine (Fig. 5A). Consistently, gentamicin uptake was lower in ΔnqrA or ΔnqrF than ATCC 33787, which was reverted by the complement of the same concentration of L-alanine (Fig. 5B). We further measured the activity of pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (KGDH), and succinate dehydrogenase (SDH) in the pyruvate cycle (the P cycle). The P cycle is a recently discovered
cycle that provides respiratory energy, including NADH for the generation of membrane potential in bacteria (23). The activity of PDH, KGDH, and SDH was reduced in the two mutants. However, when 10 mM exogenous L-alanine was added, the activity returned to normal (Fig. 5C). These results indicate that the aminoglycoside antibiotic resistance is related to the depressed alanine abundance in ΔnqrA and ΔnqrF.

We reasoned that nqrA and nqrF regulate L-alanine anabolism and/or catabolism. To test this, the expression of genes involved in L-alanine metabolism was quantified by quantitative real-time PCR (qRT-PCR). A total of 13 genes were detected. These genes degrade L-alanine to 8-amino-7-oxononanoate (bioF), L-alanyl-tRNA (alaS [N646_1642] and alaS [N646_2770]), and UDP-N-acetylmuramoyl-L-alanine (murC), transfer reversibly L-alanine to D-alanine (alr [N646_1828] and alr [N646_4376]) and pyruvate (avtA, alaA, ald, phnW, and pucG), and transform cysteine (iscS) and aspartate (asdA) to L-alanine. The increased expression of bioF and murC indicates the elevation of L-alanine catabolism, while the decreased expression of ics and asdA suggest the inhibition of L-alanine anabolism (Fig. 6). Similar gene expression was detected in lab-evolved gentamicin-resistant V. alginolyticus (VA-RGEN). Specifically, expression of asdA was decreased and expression of bioF and murC was elevated (Fig. 6A). Our recent publication showed that the P cycle is inactivated in VA-RGEN, consistent with the data that the P cycle does not provide aspartate for alanine biosynthesis (26). Thus, a shared L-alanine metabolism regulation exists between VA-RGEN and the two mutants. These results indicate that Na+/H1001-NQR disruption affects L-alanine metabolism.

Mechanisms by which nqrA and nqrF regulate L-alanine metabolism. We supposed that nqrA and nqrF regulate L-alanine metabolism through ATP since the membrane potential generated by Na+/H1001-NQR contributes to ATP formation via ATPase. ATPase activity and ATP content were reduced in ΔnqrA and ΔnqrF but recovered in the presence of 10 mM exogenous alanine (Fig. 7A and B). ATP is a substrate of cyaA for cyclic AMP (cAMP) biosynthesis. The expression of cyaA was reduced in ΔnqrA and ΔnqrF but returned to normal in the presence of 10 mM L-alanine (Fig. 7C). Consistently, the cAMP level was decreased and normal in the absence and presence of 10 mM
L-alanine, respectively, in ΔnqrA and ΔnqrF (Fig. 7D). Similarly, the reduction of crp expression was detected in the two mutants, which was recovered with the addition of 10 mM alanine (Fig. 7E). Interestingly, similar results were detected in VA-RGEN (Fig. 7A and C), suggesting a shared characteristic between VA-RGEN and ΔnqrA or ΔnqrF.

A further result showed that the loss of crp led to similar changes in gene expression as described above for ΔnqrA and ΔnqrF (Fig. 7F). These data support that Na+/H+-NQR regulates L-alanine metabolism in a cAMP/CRP-dependent manner.

L-Alanine restores the expression of other antiporter genes regulated by nqrA or nqrF. We further explored how the decreased abundance of alanine modulates the membrane potential, which contributes to the intracellular gentamicin uptake. We supposed that the decrease is related to the expression of other Na+/H+ antiporters besides Na+/NQR. To explore this, qRT-PCR was used to detect the expression of atpA/H (Atp family), and mnhD/F/G (Mnh family), nhaB (Nha family), mfe/G (RnfA family), of ATCC 33787, ΔnqrA, and ΔnqrF in the presence and absence of exogenous 10 mM L-alanine. Three types of differential expression were detected. (i) Lower expression was detected in ΔnqrA and ΔnqrF than in ATCC 33787, which was recovered due to the complement of 10 mM L-alanine, including atpA, atpE, atpH, mnhG, and rntE. (ii) Higher
expression was detected in the two mutants with or without 10 mM L-alanine, but the expression was higher in the two mutants with 10 mM L-alanine, including \( \text{atpC} \). (iii) Higher expression was detected in groups with 10 mM L-alanine than without 10 mM L-alanine, which is not related to whether \( \text{nqrA} \) or \( \Delta \text{nqrF} \) was absent, including \( \text{nhaB} \) and \( \text{rnfG} \) (Fig. 8A). Thus, the first two types are related to the action of the increased L-alanine in \( \Delta \text{nqrA} \) and \( \Delta \text{nqrF} \). Importantly, the reduced \( \text{atpA} \), \( \text{atpH} \), \( \text{mnhG} \), and \( \text{nhaB} \) and the elevated \( \text{atpC} \) overlapped between the two mutants and \( \text{VA-RGEN} \) (Fig. 8A), suggesting a shared mechanism. We further detected the expression of these differential genes of the first two types in the absence of \( \text{crp} \). Consistently, the loss of \( \text{crp} \) reduced the expression of \( \text{atpA} \), \( \text{atpE} \), \( \text{atpH} \), \( \text{mnhG} \), \( \text{rnfE} \) and elevated the expression of \( \text{atpC} \) (Fig. 8B). These results indicate that the regulation of \( \text{nqrA} \) or \( \text{nqrF} \) to other Na\(^+\)/H\(^+\) antiporters, which is under the control of cAMP-CRP, is related to L-alanine abundance and gentamicin resistance. A model showing a proposed way that Na\(^+\)-NQR confers antibiotic resistance via the regulation of L-alanine metabolism in a cAMP/CRP-dependent manner is shown in Fig. 9.

**DISCUSSION**

In the present study, we found that the absence of \( \text{nqrA} \) or \( \text{nqrF} \) leads to elevated MIC to aminoglycoside antibiotics, including amikacin, gentamicin, and kanamycin in \( \text{V. alginolyticus} \). Na\(^+\)-NQR functions as a unique redox-driven sodium pump, playing a vital role in the electron transport chain and sodium motive force (14). The sodium motive force is similar to the proton motive force (PMF), which is generated by a redox-driven proton pump out of Na\(^+\)/H\(^+\) (25, 27). It is documented that PMF contributes to aminoglycoside antibiotic uptake (25). Therefore, the elevated MIC of \( \text{nqrA} \)- and \( \text{nqrF} \)-deleted mutants to aminoglycoside antibiotics is attributed to the decrease of PMF. However, whether Na\(^+\)-NQR exhibits action through other indirect ways to regulate the generation of PMF and thereby confer antibiotic resistance is still unknown.

Na\(^+\)-NQR plays a key role in energy metabolism as a unique redox-driven sodium pump (14). Recent metabolism analysis has shown the increased reductive pathway of the tricarboxylic acid (TCA) cycle and decreased purine metabolism in \( \text{V. cholerae} \) \( \Delta \text{nqrA-F} \) mutant (17), which suggests that Na\(^+\)-NQR regulates other metabolisms.
besides the action of the Na\textsuperscript{+}-NQR pump. However, whether Na\textsuperscript{+}-NQR confers antibiotic resistance through the other metabolisms is still unknown. A line of evidence has demonstrated that the bacterial metabolic environment confounds antibiotic susceptibility and resistance (10, 23, 28). Therefore, the present study used GC-MS-based metabolomics to explore differential metabolomes due to the loss of \textit{nqrA} or \textit{nqrF} and to identify key metabolic pathways and crucial metabolites as biomarkers for further understanding of the role of Na\textsuperscript{+}-NQR in antibiotic resistance. Our results showed that the most impacted pathway is alanine, aspartate, and glutamate metabolism. Among the detected metabolites in the pathway, aspartic acid, glutamic acid, alanine, and 2-oxoglutaric acid were reduced, where the first three were identified as crucial biomarkers. Out of the three, alanine had the most absolute value of covariance \textit{p}. Besides, alanine also exhibits the most difference in abundance between ATCC 33787 and \textit{ΔnqrA} or \textit{ΔnqrF}. Thus, alanine was selected as the most impacted metabolite. These results are consistent with our recent data that the most strongly impacted KEGG pathway is alanine, aspartate, and glutamate metabolism and the decreased abundance of alanine and glutamate was observed, with the high impact on alanine in kanamycin-resistant \textit{Edwardsiella tarda} (10, 23). These findings suggest that Na\textsuperscript{+}-NQR regulates alanine, aspartate, and glutamate metabolism to cope with aminoglycoside antibiotics, where alanine is especially crucial. The conclusion is supported by the replacement of alanine with aspartate or glutamate, which showed a similar trend but lower efficacy. In addition, the alanine-mediated regulation was characterized in \textit{E. coli} \textit{nuoC, nuoF,} and \textit{nuoG}, which play a role similar to the role of the NQR complex. Therefore, aminoglycoside potentiation of alanine may be conservative in bacteria.

Compared with their parent strain ATCC 33787, \textit{ΔnqrA} and \textit{ΔnqrF} showed significant resistance to antibiotics. Our recent reports have indicated that antibiotic-resistant bacteria have an antibiotic-resistant metabolome, which can be reverted by crucial biomarkers into an antibiotic-susceptible metabolome and thereby the bacteria become sensitive to the antibiotics (10, 23). The reversion is termed a reprogramming metabolome (28). To explore the possibility that \textit{nqrA} and \textit{nqrF} confer the antibiotic resistance via a \textit{l}-alanine-related metabolome, exogenous \textit{l}-alanine was used to reprogram the resistant metabolome of \textit{ΔnqrA} and \textit{ΔnqrF} to a susceptible metabolome. Exogenous \textit{l}-alanine potentiated antibiotics to kill \textit{ΔnqrA} and \textit{ΔnqrF} by approximately 1,700- and 1,800-fold, respectively. Higher survival was detected in \textit{ΔnqrA} and \textit{ΔnqrF} than \textit{V. alginolyticus} ATCC 33787 in medium with 0.3 to 2.5 mM \textit{l}-alanine but not in medium with 5 to 20 mM \textit{l}-alanine, suggesting that the high abundance of \textit{l}-alanine reverts the resistance resulting from the loss of \textit{nqrA} and \textit{nqrF}. GC-MS analysis also demonstrated that the synergistic use of alanine and gentamicin promoted intracellular
concentration of alanine in \(\Delta nqrA\) and \(\Delta nqrF\). These results support the conclusion that \(nqrA\) and \(nqrF\) regulate \(\varepsilon\)-alanine metabolism to play a role in antibiotic resistance.

The present study further revealed that both \(nqrA\) and \(nqrF\) positively and negatively regulate gene expression of \(\varepsilon\)-alanine anabolism and catabolism, respectively. Regulation proceeds by the events that the loss of \(nqrA\) or \(nqrF\) decreases ATPase activity and ATP and cAMP levels and thereby the cAMP/CRP complex is inhibited. The inhibition reduces and promotes the transformation and degradation of \(\varepsilon\)-alanine, respectively. Similar results are obtained in \(\Delta crp\). Importantly, the changes in alanine metabolism and the cAMP/CRP complex are overlapped between \(VA-R_{\text{GEN}}\) and \(\Delta nqrA\) and \(\Delta nqrF\). The findings characterize a previously unknown \(\text{Na}^{+}\)-NQR-dependent antibiotic resistance mechanism that the reduced \(\text{Na}^{+}\)-NQR confers antibiotic resistance via reducing \(\varepsilon\)-alanine in a cAMP/CRP-dependent manner, highlighting a way to further understand the role of \(\text{Na}^{+}\)-NQR in antibiotic resistance. As noted in Results, some data are different between \(\Delta nqrA\) and \(\Delta nqrF\), such as MIC to amikacin and three reversals of the abundance of metabolites. These results suggest that the two genes are linked to their respective roles except for the shared function.

At last, the present study further explores why the reduced \(\varepsilon\)-alanine confers the antibiotic resistance due to the absence of \(nqrA\) or \(nqrF\). Exogenous \(\varepsilon\)-alanine recovers the decreased membrane potential and intracellular gentamicin concentration resulted from the loss of \(\Delta nqrA\) or \(\Delta nqrF\), suggesting that \(\varepsilon\)-alanine promotes the membrane potential via other Na\(^+\):H\(^+\) antiporters rather than \(\text{Na}^{+}\)-NQR. In \(V.\) alginolyticus cells, five Na\(^+\):H\(^+\) antiporters are included. They are NQR family, Nha family, Mnh family, Rnf family, and Atp family (29–34). The expression of Atp family \(atpA\), \(atpE\), \(aptH\) and Mnh family \(mnhG\) were lower when the NQR family did not work, most of which are overlapped in \(VA-R_{\text{GEN}}\) and are under the control of cAMP/CRP. However, exogenous 10 mM \(\varepsilon\)-alanine recovered the decrease. Similarly, expression of \(aptC\) was higher in the two mutants than control with or without 10 mM \(\varepsilon\)-alanine, but expression was higher in the two mutants with exogenous 10 mM \(\varepsilon\)-alanine than without exogenous 10 mM \(\varepsilon\)-alanine. Minato et al. found that lack of \(\text{Na}^{+}\)-NQR did not affect any of the \(\text{Na}^{+}\)-pumping-related phenotypes of \(V.\) cholerae in studying metabolism, motility, and osmotic stress resistance and speculated that other secondary \(\text{Na}^{+}\)-pump(s) can compensate for the \(\text{Na}^{+}\)-pumping activity of \(\text{Na}^{+}\)-NQR (35). These results indicate that \(nqrA\) and \(nqrF\) regulate Atp family and Mnh family via \(\varepsilon\)-alanine abundance in \(V.\) alginolyticus. Also, the \(atpA\) and \(aptC\) genes are regulated by a single promoter as an operon (36), but the differential expression is detected in \(\Delta nqrA\) or \(\Delta nqrF\). Possibly, this is related to regulation at the transcript level.

In summary, the present study showed the loss of \(nqrA\) or \(nqrF\) leads to the elevated resistance to aminoglycoside antibiotics. The losses caused similar differential metabolomes, characterizing alanine, aspartate, and glutamate metabolism and decreased alanine as the most impacted pathway and the crucial biomarker, respectively. These results indicate that the metabolic changes, especially \(\varepsilon\)-alanine metabolism, are related to the aminoglycoside resistance. \(nqrA\) and \(nqrF\) regulate \(\varepsilon\)-alanine metabolism in a cAMP/CRP-dependent manner. The regulation affects Atp family and Mnh family to generate membrane potential and promote gentamicin uptake. These findings extend our understanding of the action of \(\text{Na}^{+}\)-NQR in antibiotic resistance.

MATERIALS AND METHODS

Bacterial strain and culture conditions. Bacterial strains used in this study were from the collection of our laboratory and listed in Table S1 in the supplemental material. The antimicrobial agents amikacin, gentamicin, and kanamycin were purchased from a commercial source (Shanghai Sangon Biological Engineering Technology & Services Co. Ltd., China). A single colony was propagated in 3% NaCl Luria-Bertani (LB) (tryptone [10 g/liter], yeast extract [5 g/liter], NaCl [30 g/liter]) broth for 8 h at 30°C. The cultures were diluted to 1:100 using fresh 3% NaCl LB medium and grown at 30°C.

Construction and complementation for genetically modified mutants with \(nqrA\) or \(nqrF\) deleted. Primers were designed according to Table S2 using CE Design V1.03 software. The upstream and downstream 500-bp fragments were first amplified from the genome using two pairs of primers, primers P1 and P2 and primers P3 and P4 and then merged into a 1,000-bp fragment by overlap PCR using a pair of primers, primers P1 and P4. After the fragments were digested by SacI and XbaI, they were ligated into
the pR EI12 vector digested by the same enzymes and transformed into MC1061 competent cells. The plasmids were identified by PCR using a pair of primers, primers P1 and P4, and sequenced. The sequenced plasmids were transformed into S17 competent cells. S17 and recipient bacterium V. alginolyticus ATCC 33787 were cultured to an optical density (OD) of 1.0 and then mixed at a ratio of 4:1. After centrifugation, the mixtures were resuspended with LB medium, dropped onto sterilized filter paper soaked with LB medium, and cultured for 16 h at 30°C. All the bacteria rinsed from the filter paper with LB medium were smeared onto the LB plates with ampicillin (100 µg/ml) and chloramphenicol (30 µg/ml). After the bacteria were identified by plasmid PCR using a pair of primers P1 and P4 and sequencing, the bacteria were cultured and smeared onto the LB plates with 20% sucrose. The clones were cultured and smeared onto the LB plates with 20% sucrose or chloramphenicol. The clones, which did not grow on the LB plates with chloramphenicol but grew on the LB plates with 20% sucrose, were identified by PCR using primer P1P2, P4P7, or P5P6 for further use. For gene complementation, the entire coding regions of nqrA and nqrF were amplified by PCR and cloned into the PACYC184 plasmid. Primers are listed in Table S3. The recombinant plasmids were transformed into the ΔnqrA and ΔnqrF mutant strains and selected on Luria broth with 25 µg/ml chloramphenicol to construct the complemented mutant strains +nqrA and +nqrF.

**Measurement of MIC.** MIC was determined by antimicrobial susceptibility testing according to CLSI guidelines. In brief, the overnight bacteria cultured in 3% NaCl LB medium were diluted 1:100 (vol/vol) in fresh 0.5% yeast broth and cultured at 30°C to an optical density at 600 nm (OD600) of 0.5. Then 10 µl of 0.5 × 10⁶ CFU vswd added into each well of a 96-well microtiter polystyrene tray with 100 µl of a series of twofold dilutions of an antibiotic. The mixtures were incubated for 16 h at 30°C. MIC was defined as the lowest antibiotic concentration that inhibited visible growth. Three biological repeats were carried out.

**Measurement of the growth curve.** V. alginolyticus ATCC 33787 and its mutants ΔnqrA and ΔnqrF were separately cultured in 3% NaCl LB medium overnight and were diluted 1:100 (vol/vol) in fresh 3% NaCl LB broth. These bacteria were cultured at 30°C with shaking at 200 rpm and monitored at 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12, 14, and 24 h through measurement of OD600. All experiments were carried out in biological triplicates.

**Metabolomic analysis.** Sample preparation was performed as described previously (26). In brief, bacterial cells were collected in the exponential phase (OD600 of 0.5) by centrifugation at 8,000 rpm for 5 min at 4°C. Cellular metabolites were extracted with 500 µl of cold methanol, which contained 10 µl of 0.2 mg/ml ribitol (Sigma) as an analytical internal standard. Cells were lysed by sonication for 3 min at 30% intensity and were centrifuged for 10 min at 12,000 rpm at 4°C. Then, 500 µl of supernatant was transferred into a new 1.5-ml tube and dried in a rotary vacuum centrifuge device (Labconco). The resulting samples were used for GC-MS analysis. Each sample had five biological replicates with two technical replicates.

GC-MS analysis was carried out with a variation on the two-stage technique as previously described (37). First, protected carbonyl moieties of samples were exposed through a 90-min 37°C reaction with 80 µl of 20 mg/ml methoxyamine hydrochloride in pyridine. This was followed by the derivatization of acid proton by a 30-min 37°C reaction with the addition of 80 µl of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) (Sigma). Chemical analysis of samples was carried out by an Agilent G1701EA GC-MS ChemStation (Agilent). The injection port was maintained at 270°C. The derivatized sample of 1 µl was injected into a dodecyl benzene sulfonic acid (DBS) column (30-m length, 250-µm inner diameter (i.d.), 0.25-µm thickness) using the splitless mode. The MS source temperature was maintained at 250°C in the electron ionization (EI) (ionized directly) mode at 70 eV ionization energy and with 8,000 V acceleration voltage. The MS quad temperature was held constant at 150°C. The initial temperature of the GC oven was programmed at 85°C for 3 min, followed by an increase to 285°C at a rate of 5°C min⁻¹. Then, the temperature was increased to 310°C at a rate of 20°C min⁻¹ and held for 7 min. Helium was used as the carrier gas. The flow was kept constant at 1 ml min⁻¹. The MS was operated in a range of 50 to 600 m/z.

Multivariate statistical analyses were applied for metabolites. For each compound, a Mann-Whitney U-test was performed to detect significant differences between control and test groups. The compounds that matched by retention time and mass spectrum were merged in each sample. Correlation between the compounds was determined using the Spearman correlation coefficient. Normalization and analysis of metabolomic data were performed using Microsoft Excel. The metabolite abundance data matrix was normalized by the quantity of added internal standards and the total intensity. Z-score and hierarchical clustering were used to analyze the normalization area. Normalized data were used for hierarchical clustering in the R platform with the package “ggplot,” using the distance matrix calculated by the Euclidean method. Principal-component analysis (PCA) was used to reduce the dimension of the data set and analyze the covariance variation and emphasize the outlier in clustering. Pathway analysis was performed by MetPA, which contains pathway enrichment analysis and pathway topological analysis. Pathway enrichment analysis determines which metabolic pathways have compounds that are overrepresented and have significant perturbations to their concentrations, while MetPA employs some topological assessment tools to measure centrality or “hubness” objectively, termed pathway impact. Pathway impact is a combination of the centrality and pathway enrichment results (https://en.wikipedia.org/wiki/Metabolomic_Pathway_Analysis).

**Antibiotic bactericidal assay.** The antibiotic bactericidal assay was performed as previously described (10, 38). In brief, a single colony was propagated in 50 ml of LB broth in a 250-ml flask for 14 h at 30°C in a shaker. The cultures were collected by centrifugation at 8,000 rpm for 5 min. The samples were washed three times with 30 ml sterile saline, resuspended in M9 minimal medium (M9) supple-
mented with 10 mM acetate, 2 mM MgSO4, and 100 µM CaCl2, and then diluted to an OD600 of 0.2. Metabolite and/or antibiotics were added and incubated at 30°C and 200 rpm for 6 h. To determine bacterial counts at specified time points, 100-µl portions of samples were removed and serially diluted. An aliquot of 10 µl of each dilution was spot plated onto LB agar plates and cultured at 30°C for 12 h to determine CFU. Only dilutions that yielded 20 to 200 colonies were available. Percent survival was determined by dividing the CFU obtained from a treated sample by the CFU obtained from a control sample.

Measurement of membrane potential. Measurement of membrane potential was performed as previously described (39). In brief, a BacLight bacterial membrane potential kit (Invitrogen) was used to assess changes in membrane potential of V. alginolyticus ATCC 33787, ΔnqrA, and ΔnqrF incubated with or without 10 mM alanine. 106 CFU of bacteria were used and stained with 10 µl of 3 mM 3,3'-diethyloxacarbocyanine iodide [DIO2(3)], followed by incubation for 30 min. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using the following settings: fluorescein isothiocyanate (FITC) voltage, 250 V; mCherry voltage, 650 V; forward scatter (FSC) threshold, 1,000; recorded events, 100,000. The red/green (mCherry/FITC) values for each cell were determined, normalized, and then compared between samples. The relative PMF of test samples was determined.

Measurement of ATPase activity. To quantify intracellular F-type ATPase activity, a single colony was propagated in 50 ml LB broth in 250-ml flasks for 14 h at 30°C. Bacterial cells were collected and suspended in 250 µl of phosphate-buffered saline (PBS) and disrupted by sonic oscillation. After centrifugation, supernatants were collected. The protein concentration of the supernatant was determined by the BCA protein assay kit (Beyotime Inc., China). The activity of succinate dehydrogenase (SDH), pyruvate dehydrogenase (PDH), and α-ketoglutarate dehydrogenase (KGDH) was detected as follows. Supernatant (120 µl) containing 0.2 mg protein was transferred to a SDH reaction mix (0.5 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2.5 mM MgCl2, 6.5 mM phenazine methosulfate (PMS), 5 mM sodium succinate, 50 mM PBS) or PDH/KGDH reaction mix (0.5 mM MTT, 2.5 mM MgCl2, 6.5 mM PMS, 0.2 mM thiamine pyrophosphate [TPP], 2 mM sodium pyruvate/5 mM alpha-ketoglutaric acid potassium salt, 50 mM PBS) to a final volume of 200 µl in a 96-well plate. After incubation at 30°C for minutes, the absorbance at 566 nm was recorded. Enzyme activity was calculated according to a standard curve.

Measurement of cAMP. cAMP Complete ELISA kit (Enzo Biochem Inc., New York, NY, USA) was used to assess changes in intracellular cAMP following the instructions of the cAMP Complete ELISA kit (Enzo Biochem Inc., New York, NY, USA) was used to quantify the intracellular concentration of ATP. Cells were suspended to an OD600 of 0.6 in M9 medium and incubated with or without 10 mM α-alanine at 30°C for 6 h. A 10-ml sample with OD600 of 1.0 was dissolved in 1 ml PBS buffer (pH 7.4) and disrupted by sonication (20% output, 2-s ultrasound, and 3-s interval time for a total of 3 min) in an ice bath. Total proteins of 100 µg were added for the measurement of F-type ATPase activity with F-type ATPase activity assay kit (catalog no. GMS50248.2; Genmed, Shanghai, China). The intracellular enzyme activity unit was quantified according to the manufacturer’s manual.

Measurement of ATP. BacTiter-Glo microbial cell viability assay (Promega Corporation, Madison, WI, USA) was used to quantify the intracellular concentration of ATP. Cells were suspended to an OD600 of 0.6 in M9 medium and incubated with or without 10 mM α-alanine at 30°C for 6 h. The cell extract (50 µl) was used to determine ATP measurement by using luciferin/luciferase and BacTiter-Glo microbial cell viability assay.

Detection of intracellular gentamicin concentration. Intracellular gentamicin was detected as previously described (39). Gentamicin enzyme-linked immunosorbent assay (ELISA) rapid diagnostic kit (Beijing Clover Technology Group Inc., Beijing, China) was used to assess intracellular gentamicin concentration. Cells were suspended to an OD600 of 0.6 in M9 medium and incubated in the presence of gentamicin with or without 10 mM alanine at 30°C for 6 h. The cells were collected and washed three times with sterile saline. The resulting cells were suspended with M9 medium and adjusted to an OD600 of 1.0. An aliquot of 10 ml was sonicated for 3 min. The resulting supernatant was collected for the detection of gentamicin following the instructions of the gentamicin ELISA rapid diagnostic kit.

qRT-PCR. Quantitative real-time PCR (qRT-PCR) was carried out as described previously (40). Total RNA was isolated from V. alginolyticus using TRIzol reagent (Invitrogen Life Technologies) according to the protocol. Electrophoresis in 1% (wt/vol) agarose gels was performed to check the quality of extracted RNA. By using a PrimeScript RT reagent kit with gDNA eraser (TaKaRa, Japan), reverse transcription-PCR was carried out on 1 µg of total RNA according to the manufacturer’s instructions. Primers are listed in Table S4. qRT-PCR was performed in 384-well plates with a total volume of 10 µl, and the reaction mixtures were run on a LightCycler 480 system (Roche, Germany). Data are shown as the relative mRNA expression compared with control with the endogenous reference 16S rRNA gene.

Measurement of cAMP. cAMP Complete ELISA kit (Enzo Biochem Inc., New York, NY, USA) was used to assess the concentration of cAMP within bacterial cells. Cells were incubated in medium with or without alanine, collected, and washed three times. The resulting cells were collected for detection of cAMP following the instructions of the cAMP Complete ELISA kit.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.5 MB.
FIG S2, TIF file, 2.5 MB.
FIG S3, PDF file, 0.2 MB.
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