High Salt Cross-Protects *Escherichia coli* from Antibiotic Treatment through Increasing Efflux Pump Expression

Manlu Zhu,a Xiongfeng Dai

aSchool of Life Sciences, Central China Normal University, Wuhan, China

**ABSTRACT** Environmental stresses often co-occur when bacteria encounter antibiotic treatment inside the human body. The cellular response to environmental stresses can alter the global gene expression pattern of bacteria. However, the relationship between the cellular stress response and antibiotic susceptibility remains poorly understood. Here we studied the effect of high salt, an important environmental stress condition inside the human body, on bacterial susceptibility to antibiotics. We found that high salt reduces the susceptibility of *Escherichia coli* to tetracycline and chloramphenicol, leading to a cross-protection effect. The cross-protection effect originates from the increased AcrAB-TolC efflux pump expression level under high-salt conditions. Our study demonstrates that stress-induced gene expression alterations can cross-protect bacteria from antibiotic treatment and should thus be considered when investigating antibiotic susceptibility and applying antimicrobial treatment.

**IMPORTANCE** Environmental stresses often co-occur when bacteria confront antibiotic treatment. We provide a clear example that a natural stress condition (high salt) can cross-protect bacteria from antibiotic treatment by triggering the bacterial stress response program (elevated AcrAB-TolC efflux pump expression). Our study highlights the importance of taking the co-occurrence of bacterial environmental stresses into consideration when investigating antibiotic susceptibility and applying antimicrobial treatment.

**KEYWORDS** antibiotic susceptibility, cross-protection, efflux pumps, high salt

In nature, bacteria need to cope with many harsh environmental conditions, such as nutrient depletion, high temperature, oxidative stress, acidity, and a high salt concentration (1). Environmental stresses usually cause either a reduced growth rate or reduced viability. Under these circumstances, bacteria must initiate a stress response program to enable the survival of the population (1–4). Cellular stress response can alter the global pattern of bacterial gene expression, which is usually associated with changes in the levels of hundreds of proteins (5–8). For example, the regulon of the general stress response sigma factor RpoS contains >100 genes, which are upregulated under stress conditions (9–11).

Environmental stresses often co-occur when bacteria confront antibiotic treatment inside the human body (12). However, it remains largely unclear how the stress response program can affect the susceptibility of bacteria to antibiotic treatment. A high salt concentration is a common environmental stress condition for bacteria. The high salt contained in a “Western diet” is closely related to numerous human disorders, including hypertension, cardiovascular disease, autoimmune disease, and cognitive dysfunction (13–16). A recent study has demonstrated that the effect of a high salt concentration on the gut microbiome is closely implicated in salt-mediated human disorders (15), indicating that a high salt concentration can significantly affect the basic physiology of bacteria inside the human body. A high salt concentration imposes
hyperosmotic stress on bacteria. When confronting a high salt concentration, bacteria must initiate a stress response to tackle the severe loss of water and turgor pressure (2, 17–20). Cellular stress response enables *Escherichia coli* to accumulate osmolytes such as potassium, glutamate, and trehalose to maintain the balance of external and internal osmolarity (21). This process helps *E. coli* regain water and turgor pressure to support growth.

Though a high salt concentration is proposed to significantly affect microbial physiology, it remains unclear whether a high salt concentration can affect the antibi-otic susceptibility of bacteria. Here, we find that a high salt concentration cross-protects *E. coli* from antibiotic treatment, which results from increased AcrAB-TolC efflux pump expression under high-salt conditions.

**RESULTS AND DISCUSSION**

**Decreased antibiotic susceptibility of *E. coli* under high-salt conditions.** To investigate the effect of high-salt conditions on antibiotic susceptibility, we measured the antibiotic growth inhibition curve of *E. coli* cells under both normal conditions (0.1 M NaCl in glucose minimal medium) and high-salt conditions (0.4 M NaCl in glucose minimal medium). Two well-known ribosome-targeting antibiotics, tetracycline (binds to the 30S ribosomal subunit) and chloramphenicol (binds to the 50S ribosomal subunit), were used to study drug susceptibility (22). The exponential growth rate of *E. coli* cells was plotted as a function of the antibiotic concentration in the medium. As shown in Fig. 1A, the bacterial growth rate dramatically drops with increasing concentrations of tetracycline under normal conditions (red symbols). Under high-salt conditions, though the growth rate is much lower than that under normal conditions in drug-free medium (0.52/h at 0.4 M NaCl versus 0.96/h at 0.1 M NaCl), it drops very slowly with increasing concentrations of tetracycline (purple symbols). The growth rate under high-salt conditions exceeds that under normal conditions when the tetracycline concentration is >1 μM. This result demonstrates that high salt can lead to cross-protection against antibiotics. For a direct comparison, we made the inhibition curve of the relative growth rate (the growth rate is normalized by the growth rate in drug-free medium).
medium, $\lambda/\lambda_0$ (Fig. 1B). The tetracycline susceptibility of *E. coli* under high-salt conditions is indeed much weaker than that under normal conditions. The bacterial growth rate drops by ~60% at 1 $\mu$M tetracycline under normal conditions, while under high-salt conditions, it drops by only ~35%, even at 4 $\mu$M tetracycline. Similarly, high salt also reduces the chloramphenicol susceptibility of *E. coli* (Fig. 1C and D). The cross-protection effect against chloramphenicol is especially remarkable in glucose-6-phosphate minimal medium, where 4 $\mu$M chloramphenicol has no effect on the bacterial growth rate under high-salt conditions (Fig. S1).

Significant alterations in efflux pump and porin protein expression levels under high-salt conditions. To investigate the origin of cross-protection against antibiotics mediated by high salt, we postulated that high salt could change the level of some specific proteins, which ultimately reduced the intracellular drug concentration. In principle, intracellular drug accumulation can be affected by drug permeation and drug efflux (23). Porin proteins OmpF and OmpC are among the most abundant outer membrane proteins and are proposed to be responsible for controlling drug influx into cells. On the other hand, AcrAB-ToIC is the major multidrug efflux pump system that pumps a drug out of the cell to reduce its effective intracellular concentration (23–25). We applied quantitative mass spectrometry and real-time quantitative PCR (qPCR) to measure relative *ompF*, *ompC*, *acrA*, *acrB*, and *toIC* expression in normal and high-salt media. As shown in Fig. 2, the relative *acrA*, *acrB*, and *toIC* expression levels all strongly increase under high-salt conditions. The expression levels of two porin proteins exhibit the opposite trend under high-salt conditions. *ompF* expression strongly decreases while *ompC* expression increases remarkably under high-salt conditions. Overall, on the basis of the above-described results, the reduced antibiotic susceptibility that occurs under high-salt conditions may be attributed to increased drug efflux, decreased drug influx (mediated by OmpF), or both.

Disappearance of cross-protection effect in efflux pump mutants. We next characterized the antibiotic susceptibility of *E. coli* mutants deficient in either efflux pump or porin proteins (Fig. 3 and 4). Both the *acrB* and *toIC* mutants exhibit remarkably higher susceptibility to tetracycline and chloramphenicol than wild-type cells under both normal and high-salt conditions, confirming the important role of the
AcrAB-TolC efflux pump in bacterial drug resistance. Strikingly, unlike the case of wild-type cells, the growth rates of acrB and tolC mutant cells under high-salt conditions also dramatically drop with increasing concentrations of tetracycline and chloramphenicol (Fig. 3A and C). The disappearance of the cross-protection effect is explicitly demonstrated in the relative growth rate inhibition curve; the relative growth rate inhibition curves of the acrB and tolC mutants under both normal and high-salt conditions almost completely overlap (Fig. 3B and D). This result indicates that the AcrAB-TolC efflux pump is related to the cross-protection against antibiotics mediated by high-salt conditions.

The growth inhibition curve of the ompC mutant was almost the same as that of wild-type cells (Fig. 4), indicating that the change in ompC expression is not related to the cross-protection effect under high-salt conditions. The ompF mutant is slightly less susceptible to tetracycline treatment than wild-type cells under both normal and high-salt conditions (Fig. 4A). However, the growth rate of the ompF mutant still exhibits a strong dependence on the tetracycline concentration under normal condi-
Moreover, the chloramphenicol susceptibility of the ompF mutant is similar to that of the ompC mutant and the wild-type strain under both normal and high-salt conditions (Fig. 4B). This indicates that the reduction of ompF expression under high-salt conditions contributes only marginally to cross-protection against antibiotics.

**Decreased antibiotic susceptibility of efflux pump overexpression strain.** To further confirm that the cross-protection effect originates from the increased AcrAB-TolC efflux pump expression level, we studied whether artificial AcrAB-TolC efflux pump overexpression could also lead to decreased antibiotic susceptibility under normal conditions. We constructed an artificial AcrAB-TolC efflux pump overexpression vector (Fig. 5A). The acrB operon was placed downstream of the P_tac promoter. In addition, the tolC gene, together with its native ribosome-binding site (RBS), was directly inserted downstream of the acrAB operon to form a single acrAB-tolC cistron. Therefore, the expression of acrAB-tolC is driven by the inducible P_tac promoter, which is under the regulation of a p_lacI_q-lacI cassette. The AcrAB-TolC overexpression vector pZE-acrAB-tolC was transformed into acrB and tolC mutants to obtain the FL15 and FL16 strains, respectively. We then measured the antibiotic growth inhibition curves of the FL15 and FL16 strains under normal conditions.

As shown in Fig. 5 and S2, elevated AcrAB-TolC efflux pump expression (20 μM isopropyl-β-D-thiogalactopyranoside [IPTG]) indeed reduces the antibiotic susceptibility of E. coli under normal conditions, mimicking the pattern of the growth inhibition curve of wild-type cells under high-salt conditions. Even in the absence of an inducer, the leaky AcrAB-TolC efflux pump expression driven by the P_tac promoter has mildly reduced the antibiotic susceptibility compared with that of wild-type cells. On the contrary, the FL30 and FL31 strains, which overexpress green fluorescent protein, used as the control are still as highly susceptible to antibiotic treatment as the acrB and tolC mutants. Overall, the above results strongly support the notion that the antibiotic cross-protection effect of high-salt conditions results from increased AcrAB-TolC efflux pump expression.
The functional AcrAB-ToIC multidrug efflux pump requires all three proteins (25); therefore, the increased antibiotic tolerance should, in principle, require increased expression of all three efflux pump proteins, as found under high-salt conditions (Fig. 2). In support of this assumption, both the FL17 (acrB deficient and overexpressing AcrA and AcrB) and FL18 (tolC deficient and overexpressing ToIC) strains fail to obtain higher antibiotic tolerance than wild-type cells (Fig. S3 and S4). This result indicates that it is the increase in all three efflux pump proteins (functional AcrAB-ToIC efflux pump) that is indispensable for the antibiotic cross-protection effect of high-salt conditions.

**Conclusion.** Our study demonstrates that a high salt concentration, a common environmental stress condition encountered by bacteria inside the human body, can cross-protect them from antibiotic treatment by triggering the stress response program. It was known before that nutrient limitation can also enhance bacterial antibiotic tolerance by increasing the fraction of persister cells in the bacterial population (26–29). The increased fraction of persister cells originates from the induction of toxin-antitoxin activity by ppGpp signaling under nutrient-limited conditions. The persister cells represent a subpopulation that can survive antibiotic treatment while they remain largely metabolically dormant. Instead, here we find that high-salt conditions can reduce bacterial susceptibility to antibiotics at the whole-population instead of the subpopulation level. Our study highlights the importance of taking the co-occurrence of environmental stressors into consideration when investigating antibiotic susceptibility and applying antimicrobial treatment. The antibiotic cross-protection mediated by environmental stresses inside the human body may accelerate the spread of bacterial drug resistance, which will further complicate the current severe drug resistance situation and should be taken into consideration in future clinical studies.

**MATERIALS AND METHODS**

**Strains.** The strains used in this study included the wild-type K-12 NCM3722 strain (22, 30) and its derivatives including acrB, tolC, ompF, and ompC mutants and AcrAB-ToIC overexpression strains FL15 and FL16. To construct the efflux pump- and porin protein-deficient forms of strain NCM3722, the acrB:kan, tolC:kan, ompF::kan, and ompC::kan alleles in related mutant strains from the Keio collection (31) were transferred into NCM3722 through P1 transduction to generate the four mutant strains, respectively.

To construct the AcrAB-ToIC overexpression vector pZEA-acrAB-tolC, a plasmid-tolC cassette was first inserted into the AattI/Xhol sites of the pZE11-luc vector (32); a P
\_mar promoter was PCR amplified and cloned into the Xhol/KpnI sites to replace the P
\_mar promoter of the native vector; the coding sequence of the acrB operon was then inserted downstream of the P
\_mar promoter at the KpnI/XbaI sites, resulting in pZEA-acrAB; and finally, the coding sequence of the tolC gene, together with its RBS, was inserted into the XbaI sites of pZEA-acrAB to form a single cistron with the acrAB operon, generating the pZEA-acrAB-tolC vector. The pZEA-acrAB-tolC vector was then transformed into the acrB and tolC mutant strains to generate the FL15 and FL16 strains, respectively. The PCR reagents used in this study were Golden PCR mix (green) and TS super PCR mix (Tsingke BioTech Co., China).

The pZEA-acrAB vector was transformed into the acrB:kan mutant strain, resulting in the FL17 strain. The acrB operon in the pZEA-acrAB vector was also replaced with the tolC and gfp genes, resulting in the pZEA-toIC and pZEA-gfp vectors, respectively. The pZEA-toIC vector was transformed into the tolC:kan mutant strain to obtain the FL18 strain. The pZEA-gfp vector was transformed into the acrB and tolC mutants to generate the FL30 and FL31 strains.

**Growth medium.** The growth medium used in this study was either morpholinepropanesulfonic acid (MOPS)-buffered glucose minimal medium (used in most of our experiments) or glucose-6-phosphate minimal medium (used solely in the experiment described in Fig. S1) as described by Cayley et al. (17). The final NaCl concentrations in normal and high-salt media were 0.1 and 0.4 M, respectively. The growth media were supplemented with different concentrations of tetracycline and chloramphenicol (Solarbio Life Sciences, Beijing). The LB medium used to grow seed cultures contained 0.5% yeast extract, 1% tryptone, and 1% sodium chloride (33).

**Cell growth.** Cell growth experiments were always performed in a 37°C air bath shaker (220 rpm). Seed culture was grown in LB medium (Coolaber Biotech, Beijing) for several hours and inoculated into the minimal medium supplemented with antibiotics for overnight growth as precultures. On the next day, the precultures were inoculated into the same antibiotic-containing minimal medium at an initial optical density at 600 nm (OD\_600 of ~0.01 as the final experimental culture. During the cell growth procedures, six to eight OD\_600 data points in the range of 0.05 to 0.5 were measured by a GENESYS 30 visible spectrophotometer (Thermo Fisher Scientific) at different time points to generate an exponential-growth curve for calculation of the bacterial growth rate.

**Real-time qPCR.** Two milliliters of cell culture (OD\_600 of ~0.4) was used for total RNA extraction with the RNAprep Bacterial kit (Tiangen, China). The total RNA concentration was then measured by NanoDrop spectrophotometry. A 1-μg sample of total RNA was used for cDNA synthesis via reverse tran-
scription with Transcriptor cDNA Synthesis SuperMix (Tiangen, China). The qPCRs were performed with a SuperReal Premix SYBR green Plus kit (Yeasen Biotech, Shanghai, China) in accordance with the manual. The qPCRs were carried out in a Bio-Rad CFX96 Touch real-time PCR system with the following protocol: 95°C for 15 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The ftsZ housekeeping gene was used as the internal reference.

Measurement of protein abundance. The abundances of porin and efflux pump proteins were measured by quantitative mass spectrometry as described by Hui et al. and Dai et al. (5, 20).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00095-18.

FIG S1, PDF file, 0.1 MB.

FIG S2, PDF file, 0.04 MB.

FIG S3, PDF file, 0.1 MB.

FIG S4, PDF file, 0.1 MB.

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X.D. designed the research, M.Z. performed the experiments, and X.D. and M.Z. analyzed the data and wrote the paper together.

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