Characterization of membrane transporters involved in metabolite transport in lactic acid bacteria

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Research objective

Prokaryotic drug efflux pumps, which are responsible for expelling various molecules from the cell, have been extensively studied in the context of antibiotic resistance. In gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, resistance-nodulation-division (RND)-type efflux pumps (e.g., *E. coli* AcrAB-TolC), which require the presence of an outer membrane protein for proper function, have been implicated in conferring high-level resistance to clinical isolates and are important targets for developing novel therapeutic strategies to treat bacterial infections.

Gram-positive bacteria, which include lactic acid bacteria, do not possess RND-type efflux pumps because they lack an outer membrane protein. However, these bacteria still encode efflux pumps belonging to the major facilitator superfamily as well as the multidrug and toxin extrusion, drug/metabolite transporter, and ATP-binding cassette (ABC) families.[1)] ABC transporters use ATP hydrolysis to drive substrate transport. Most of these efflux pumps remain poorly characterized because they are not associated with drug resistance phenotypes, leaving their functions largely unknown.

Prior to this study, a pneumococcal efflux pump overexpression library comprising 30 different strains was generated to comprehensively assess the role of efflux pumps in antibiotic resistance. Screening of this library identified the ABC transporters PatAB and BceAB, which were previously shown to play a role in antibiotic resistance. Additionally, a previously uncharacterized ABC transporter, FoeAB, was found to confer resistance to fosfomycin, which targets the cell wall synthesis pathway. This heterodimeric transporter consisting of FoeA and FoeB subunits is widely distributed among streptococcal and enterococcal species.

The paucity of hits from this screening suggests that pneumococcal drug efflux pumps fulfill physiologically important roles beyond antibiotic extrusion. For example, FoeAB is unlikely to function solely as a fosfomycin exporter and may play a role in transporting physiological substrates related to cell survival or homeostasis. Fosfomycin, which is a phosphonic acid derivative containing an epoxypropyl group, inhibits the cell wall synthesis enzyme MurA by acting as a structural analog of its native substrate, phosphoenolpyruvate. This indicates that FoeAB is capable of transporting substrates containing phosphate groups.

In this study, we characterized the functions and structure of FoeAB from the lactic acid bacterium *Streptococcus thermophilus*. Additionally, to elucidate the cellular role of FoeAB, we generated *foeAB*-knockout and -overexpressing strains and compared the metabolic profiles of their culture supernatants to identify their physiological substrates.

Methods

S. thermophilus FoeA containing a Twin-Strep-tag at the C terminus was co-expressed with FoeB in *E. coli* C43(DE3) cells, and the membrane fraction was solubilized using either *n*-dodecyl-β-D-maltoside (DDM) or lauryl maltose neopentyl glycol (LMNG). Then, FoeAB was purified using affinity and size-exclusion chromatographies.

The DDM-solubilized FoeAB samples were used for structural analysis. To stabilize the desired conformation, nucleotides were added prior to grid preparation. The samples were imaged using cryo-electron microscopy (cryo-EM), and three-dimensional constructs were generated.

To evaluate the transport activity of FoeAB, the LMNG-solubilized fraction was reconstituted in phosphatidylcholine liposomes. FoeAB-dependent transport activity was initiated by adding various substrates and GTP. After the removal of exogenous substrates, the substrates that were taken up into the liposomes were quantified using mass spectrometry.

Metabolomic analysis was performed on the culture supernatants of *Streptococcus pneumoniae* FoeAB-knockout and -overexpressing strains cultured in artificial media. The putative physiological substrates were identified by comparing the resulting metabolomic profiles of the two strains.

Results

ABC-type efflux pumps alternate between their inward- and outward-facing conformations. To elucidate the substrate transport mechanism, we determined both conformations of FoeAB using cryo-EM at the resolutions of 2.8–3.3 Å. The samples prepared without any additives or in the presence of the ATP analog adenylyl imidodiphosphate (AMP-PNP) displayed an inward-facing conformation. Although this conformation should accommodate substrates in the transmembrane domain, no additional density corresponding to fosfomycin was observed when it was added to the samples. Instead, additional density corresponding to phosphate or sulfate ions was observed, suggesting that the presence of this molecule inhibited fosfomycin binding (Figure 1).

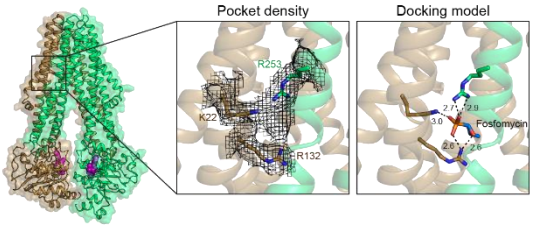

Figure 1. Inward-facing structure of FoeAB with close-up views of the substrate-binding site and a fosfomycin-docking model.

Basic residues surrounded this additional density, indicating that they interacted with fosfomycin phosphate. *E. coli* cells overexpressing FoeAB mutants with alanine substitutions in these residues lost their fosfomycin resistance capability, supporting the role of these residues in substrate recognition. Additionally, molecular docking studies revealed that fosfomycin can occupy this pocket and interact with the basic residues.

To acquire an outward-facing conformation, ATP was added to an FoeAB mutant in which the ATPase activity had been compromised. No additional density was observed in the binding pocket in this conformation, suggesting that FoeAB underwent a conformational transition that allowed for substrate release. Comparison of the binding pockets of the two conformations revealed that FoeA Arg136 played a role in stabilizing the position of FoeB Arg253 in the inward-facing state, enabling its participation in phosphate recognition. By contrast, this organization was disrupted in the outward-facing conformation, which may have promoted substrate release.

The NTPase and transport activities were assessed using FoeAB reconstituted in phosphatidylcholine liposomes. The reconstituted protein exhibited ATPase activity, confirming its successful incorporation into the liposomes. Intriguingly, FoeAB exhibited better GTPase activity than ATPase activity, suggesting that it selectively uses GTP to mediate substrate transport.

To measure the transport activity of FoeAB, the amount of fosfomycin transported into liposomes via the ABC transporter was quantified using mass spectrometry (Figure 2). Wild-type FoeAB showed transport activity. By contrast, the FoeA R132A mutation that disrupts substrate recognition completely abolished the transport activity. The FoeA G126D mutation, which increases fosfomycin resistance when expressed in *E. coli*, resulted in a marked increase in fosfomycin transport. Taken together, these results support a direct correlation between the transport activity of FoeAB and its role in drug resistance.

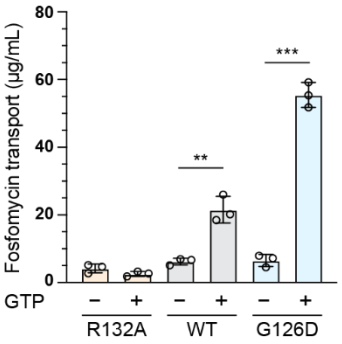

Figure 2. *In vitro* quantification of FoeAB-mediated fosfomycin transport into liposomes.

The structural analysis suggested that FoeAB is capable of extruding phosphorylated metabolites. To test this hypothesis, metabolomic analysis was performed on the culture supernatants of *S. pneumoniae foeAB*-knockout and -overexpressing cells. The results revealed a glycolytic intermediate that was present only in the *foeAB*-overexpressing sample, suggesting that this molecule is a physiological substrate of FoeAB.

Conclusion

In this study, we successfully elucidated the structures of FoeAB in both inward- and outward-facing conformations, thereby revealing how this ABC-type efflux pump extrudes

fosfomycin from bacterial cells. Additionally, we identified a putative physiological substrate of FoeAB using metabolomics. In the future, we will use the transport assay established in this study to measure the transport of candidate substrates by FoeAB and clarify its physiological roles, which will also provide valuable insights into the purpose of drug efflux pumps in bacterial cells.

References

1) Du, D. et al. (2018) Multidrug efflux pumps: structure, function and regulation. *Nat. Rev. Microbiol.* **16:** 523–539.