



AcrB, AcrD, and MdtABC Multidrug Efflux Systems Are Involved in Enterobactin Export in *Escherichia coli*

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Abstract

Escherichia coli produces the iron-chelating compound enterobactin to enable growth under iron-limiting conditions. After biosynthesis, enterobactin is released from the cell. However, the enterobactin export system is not fully understood. Previous studies have suggested that the outer membrane channel TolC is involved in enterobactin export. There are several multidrug efflux transporters belonging to resistance-nodulation-cell division (RND) family that require interaction with TolC to function. Therefore, several RND transporters may be responsible for enterobactin export. In this study, we investigated whether RND transporters are involved in enterobactin export using deletion mutants of multidrug transporters in *E. coli*. Single deletions of *acrB*, *acrD*, *mdtABC*, *acrEF*, or *mdtEF* did not affect the ability of *E. coli* to excrete enterobactin, whereas deletion of *tolC* did affect enterobactin export. We found that multiple deletion of *acrB*, *acrD*, and *mdtABC* resulted in a significant decrease in enterobactin export and that plasmids carrying the *acrAB*, *acrD*, or *mdtABC* genes restored the decrease in enterobactin export exhibited by the Δ *acrB acrD mdtABC* mutant. These results indicate that AcrB, AcrD, and MdtABC are required for the secretion of enterobactin.

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Introduction

Multidrug efflux transporters cause serious problems in cancer chemotherapy and in the treatment of bacterial infections. In gram-negative bacteria, transporters belonging to the resistance-nodulation-cell division (RND) family are particularly effective in generating resistance because they form a tripartite complex with periplasmic proteins and an outer membrane protein channel. The RND transporters have wide substrate specificity [1]. The AcrAB–TolC system is composed of the RND transporter AcrB, membrane fusion protein AcrA, and multifunctional outer membrane channel TolC. It has been suggested that the AcrAB–TolC multidrug efflux system is capable of capturing substrates in the periplasm rather than in the membrane or the cytoplasm [2]. This claim is supported by high-resolution structures of AcrB, in which access pathways from the periplasm, but not from the cytoplasm, have been identified [3,4].

TolC plays an important role in the excretion of a wide range of molecules in *E. coli*, including antibiotics [5–7], bile salts [8–10], organic solvents [11], several antibacterial peptides such as colicin V [12,13] and microcin J25 [14] and a large protein toxin, α -hemolysin [15,16]. TolC interacts with a variety of inner membrane transporters and enables *E. coli* to expel structurally diverse molecules. In *E. coli*, AcrB, AcrD, AcrEF, MdtABC, and MdtEF belong to the RND transporters and require TolC to function [7,17].

Iron is an essential element for many biological processes, such as amino acid and nucleotide synthesis, electron transport, and peroxide reduction [18]. Many bacteria excrete iron-chelating compounds called siderophores to grow under iron-limited conditions. *E. coli* can produce the catecholate siderophore enterobactin (also called enterochelin), which is a cyclic triester of 2, 3-dihydroxybenzoylserine (DHBS) [19,20]. Enterobactin is synthesized from chorismate in the cytoplasm [21] and exported from the cell. Extracellular iron-loaded enterobactin is taken up via the outer membrane receptor FepA and translocated to the periplasm [22,23]. Fe-enterobactin is chaperoned by FepB to the ATPase-dependent transporter FepDGC and shuttled to the cytoplasm. In the cytoplasm, enterobactin is degraded by Fes esterases to release iron [24–27].

The systems responsible for enterobactin synthesis and uptake are well characterized, as presented above. In contrast, the enterobactin export system is not fully understood. Previously, enterobactin export across the cytoplasmic membrane was shown to be dependent on the major-facilitator transporter EntS (the *ybdA* gene product) [28]. EntS has also been shown to be responsible for enterobactin export in *Salmonella enterica* serovar Typhimurium [29]. Bleuel *et al.* showed that the outer membrane channel TolC is involved in enterobactin export from the periplasm to the culture medium [30]; however, the RND transporters required for enterobactin export have not yet been identified. The RND transporters have wide substrate specificity

Table 1. Strains and plasmids used in this study.

Strain or Plasmid	Original name	Characteristics	Source or references
Strains as in text			
Wild-type	MG1655	<i>Escherichia coli</i> wild type	31
Δ <i>acrB</i>	NKE96	Δ <i>acrB</i>	36
Δ <i>acrD</i>	NKE94	Δ <i>acrD</i> ::Cm ^R	37
Δ <i>mdtABC</i>	NKE133	Δ <i>mdtABC</i> ::Km ^R	37
Δ <i>acrEF</i>	NKE129	Δ <i>acrEF</i> ::Km ^R	This study
Δ <i>mdtEF</i>	NKE138	Δ <i>mdtEF</i> ::Km ^R	34
Δ <i>tolC</i>	NKE95	Δ <i>tolC</i> ::Cm ^R	35
Δ <i>entS</i>	NKE869	Δ <i>entS</i>	This study
Δ <i>acrB acrD</i>	NKE126	Δ <i>acrB</i> Δ <i>acrD</i>	37
Δ <i>acrB mdtABC</i>	NKE141	Δ <i>acrB</i> Δ <i>mdtABC</i> ::Km ^R	37
Δ <i>acrD mdtABC</i>	NKE1288	Δ <i>mdtABC</i> ::Km ^R Δ <i>acrD</i> ::Cm ^R	This study
Δ <i>acrB acrD mdtABC</i>	NKE1317	Δ <i>acrB</i> Δ <i>acrD</i> Δ <i>mdtABC</i>	37
Δ <i>acrB acrD mdtABC mdtEF</i>	NKE1327	Δ <i>acrB</i> Δ <i>acrD</i> Δ <i>mdtABC</i> Δ <i>mdtEF</i>	This study
Δ <i>acrB acrD mdtABC mdtEF acrEF</i>	NKE1329	Δ <i>acrB</i> Δ <i>acrD</i> Δ <i>mdtABC</i> Δ <i>mdtEF</i> Δ <i>acrEF</i> ::Km ^R	This study
Δ <i>acrB acrD mdtABC/vector</i>	NKE1575	Δ <i>acrB</i> Δ <i>acrD</i> Δ <i>mdtABC</i> /pTrc99A	This study
Δ <i>acrB acrD mdtABC/pacrAB</i>	NKE1576	Δ <i>acrB</i> Δ <i>acrD</i> Δ <i>mdtABC</i> /pacrAB	This study
Δ <i>acrB acrD mdtABC/pacrD</i>	NKE1578	Δ <i>acrB</i> Δ <i>acrD</i> Δ <i>mdtABC</i> /pacrD	This study
Δ <i>acrB acrD mdtABC/pmdtABC</i>	NKE1583	Δ <i>acrB</i> Δ <i>acrD</i> Δ <i>mdtABC</i> /pmdtABC	This study
Vector			
pKD4		rep _{R6K7(p)} Ap ^R FRT Km ^R FRT	33
pCP20		rep _{p5C101} ¹⁵ Ap ^R Cm ^R <i>cl857</i> λ.P _R flp	33
pTrc99A		Vector, Ap ^R	Amercham Pharmacia Biotech
Plasmids			
<i>pacrAB</i>		<i>acrAB</i> genes cloned into pTrc99A, Ap ^R	This study
<i>pacrD</i>		<i>acrD</i> genes cloned into pTrc99A, Ap ^R	This study
<i>pmdtABC</i>		<i>mdtABC</i> genes cloned into pTrc99A, Ap ^R	This study

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and require TolC for their function. Therefore, it is possible that the RND transporters are responsible for enterobactin export. In this study, we investigated whether RND transporters are involved in enterobactin export using several deletion mutants of RND transporter genes and high-performance liquid chromatography analysis.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* strains used in this study are derived from the wild-type strain MG1655 [31]. For production and detection of enterobactin, strains were grown at 37°C for 13 h with shaking in iron-restricted T medium containing the following per liter: 5.8 g NaCl, 3.7 g KCl, 0.113 g CaCl₂, 0.1 g MgCl₂·6H₂O, 1.1 g NH₄Cl, 0.272 g KH₂PO₄, 0.142 g Na₂SO₄, and 12.2 g Tris. The pH was adjusted to 7.4 with 1 N HCl, and medium was supplemented with 33.4 ml deferrated casamino acids, 0.4% (wt/vol) glucose, 0.005% (wt/vol) nicotinic acid, 1% (wt/vol) thiamine before inoculation, and 150 μM 2,2'-dipyridyl. Deferrated casamino acids were prepared using the following methods. Twenty-five ml of 10% (wt/vol) casamino acids were mixed with 0.75 g hydroxyquinoline and 25 ml chloroform. Thereafter, the

supernatant was again mixed with 25 ml chloroform and its supernatant was collected as deferrated casamino acids. Aliquots of iron-restricted T medium were inoculated with 0.1% (v/v) of overnight cultures of strains grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) [28,30,32]. Ampicillin was added to the growth medium at the final concentration of 100 mg/L for plasmid maintenance.

Construction of gene deletion mutants

To construct the drug efflux mutants, gene disruption was performed as described by Datsenko and Wanner [33]. The mutants Δ *tolC*, Δ *acrB*, Δ *acrD*, Δ *mdtABC*, Δ *mdtEF*, Δ *acrB acrD*, Δ *acrB mdtABC*, and Δ *acrB acrD mdtABC* were constructed as previously described [34–37]. The oligonucleotide primers *acrE*-P1 (TTGGGTAATAACGCGCTTTTGGTTTTITGAG-GAATAGTAGTGTAGGCTGGAGCTGCTTC) and *acrF*-P2 (AAATAATAAAGGCACCCGAAAGCGCTTTATGTTTCT-GATCATATGAATATCCTCCTTAG) were used to construct the Δ *acrEF* mutant. The kanamycin resistance gene *aph*, flanked by F1p recognition sites, was amplified by polymerase chain reaction (PCR) using the primers listed above. The resulting PCR products were used to transform the recipient MG1655 strain harboring the plasmid pKD46, which expresses lambda Red recombinase. The chromosomal structures of the mutated loci

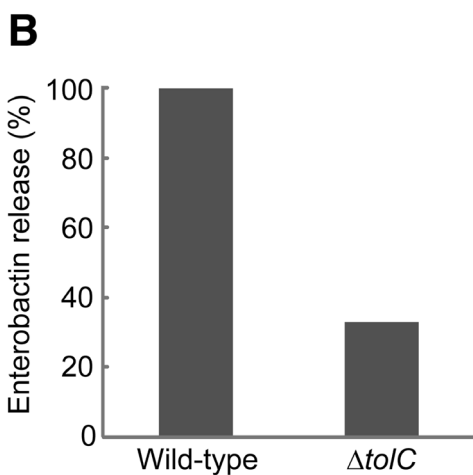
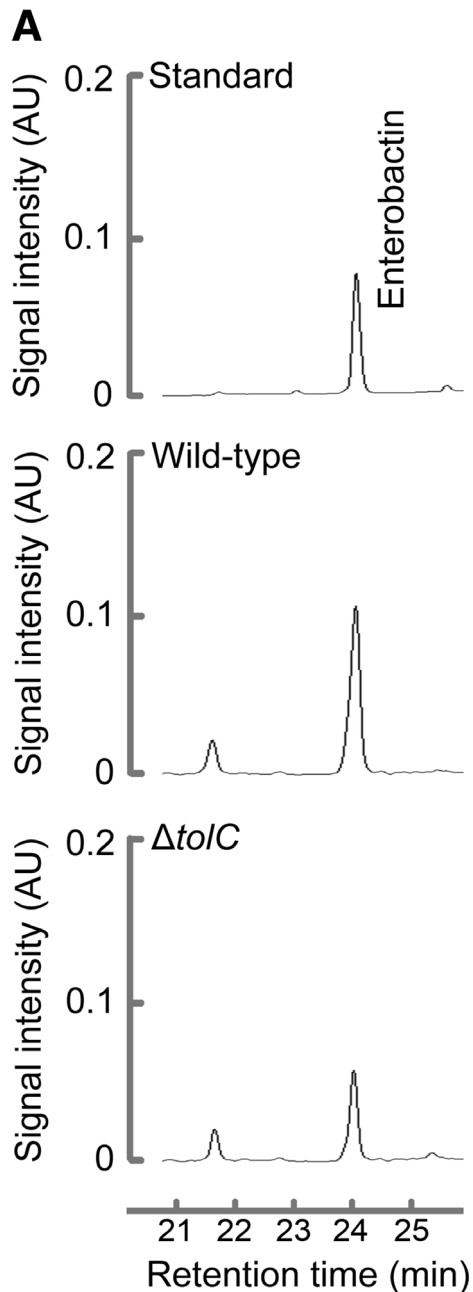


Figure 1. RP HPLC analysis of enterobactin release from the wild-type strain and the $\Delta tolC$ mutant. (A) Chromatograms of enterobactin prepared from the supernatants of the wild-type strain and the $\Delta tolC$ mutant. Peaks were identified using HPLC-grade standards. (B) The amount of enterobactin exported by $\Delta tolC$ mutant relative to that exported by the wild-type was calculated using the peak areas. The peak area corresponding to enterobactin released from the wild-type strain was defined as 100%. doi:10.1371/journal.pone.0108642.g001

were verified by PCR using the primers *acrE-F* (GTAAAA-TAAATAATATATATTATTACCTA) and *acrF-R* (CGTGAGCACAGCCCGCCAGCAATGCGGTGA), and K-1 (CAGTCATAGCCGAATAGCCT) and K-2 (CGGTGCCCTGAATGAACTGC). To construct $\Delta acrD$ *mdtABC*, $\Delta acrB$ *acrD* *mdtABC* *mdtEF*, and $\Delta acrB$ *acrD* *mdtABC* *mdtEF* *acrEF* mutants, the individual deletions were transferred to a fresh isolate of MG1655 by P1 transduction. The *cat* or *aph* genes were eliminated using plasmid pCP20, as previously described [33]. To construct the $\Delta entS$ mutant, precise in-frame deletions were generated using crossover PCR. The oligonucleotide primers *entS-No* (CGCGGATCCAAAGGCAACAAT-CAATGAGGC) and *entS-Ni* (CACGCAATAACCTTCA-CACTCCAAATTTATAACCATTACAATGCCTTGCCATC), plus *entS-Ci* (GTTATAAATTTGGAGTGTGAAGGT-TATTGCGTGTAATGCTTAAAACAGCGCCTTAAGCC), and *entS-Co* (CGCGTCGACGACGACAAAACGTGG-CAGTC) were used. Then the fragment containing the deletion was cloned into the *Bam*HI site of vector pKO3, after which the deletion was introduced into the chromosome by gene replacement, as previously described [38].

Construction of plasmids

The *acrAB* and *acrD* genes were subcloned from pHS*GacrAB* and pHS*GacrD* into vector pTrc99A using *Bam*HI and *Hind*III, and *Hind*III and *Sac*I, respectively [7]. PCR with PrimeSTAR GXL DNA polymerase (TaKaRa Bio Inc., Otsu, Japan) and the primers *mdtABC-EcoRI* (CGCGAATTCAA-GAAATCCTTTCTTAGAGAA) and *mdtABC-KpnI* (CGCGGTACCTTACTCGGTTACCGTTTGTTT) was used to amplify *mdtABC* from the chromosomal DNA of *E. coli* MG1655. This process introduced the restriction enzyme recognition site present in the multicloning region of the pTrc99A vector. The DNA fragments were digested with restriction enzymes and then ligated into the multicloning region of pTrc99A.

Extraction of enterobactin

Enterobactin was prepared from supernatants separated from approximately 10^9 cells of each strain. Supernatants of 13 h cultures were acidified with 50 μ l 12 N HCl per 10 ml and extracted twice with 5 ml ethyl acetate. Thereafter, the ethyl acetate phase was concentrated by evaporation (Buchi, Postfach, Switzerland). Dried residues were resuspended in 500 μ l methanol and analyzed by reverse-phase (RP) high-performance liquid chromatography (HPLC).

HPLC analysis

RP HPLC analysis was performed on a Symmetry C18 Column (C₁₈, 4.6 \times 250 mm, 5 μ m: Waters Corp, Milford, MA, USA) using a LaChrom Elite instrument (Hitachi, Tokyo, Japan) containing a D-2000 interface, a L-2130 pump, and a L-2400 UV detector. The mobile phase consisted of 0.075% (vol/vol) trifluoroacetic acid in H₂O (pH 2) and acetonitrile. The flow rate was adjusted to 1 ml min⁻¹, and 20 μ l of each supernatant extraction was injected and separated as described by the

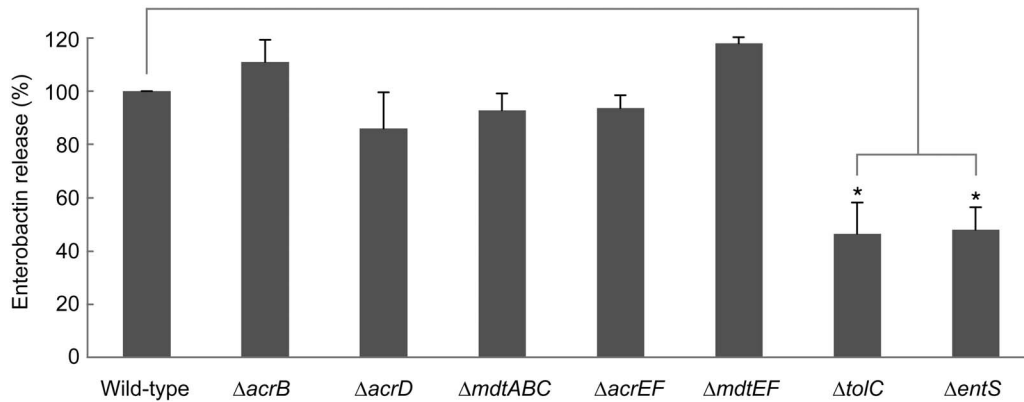


Figure 2. RP HPLC analysis of enterobactin released from deletion mutants of the RND-type efflux system genes. The amounts of enterobactin exported by deletion mutants calculated using each peak area are shown. The peak area corresponding to enterobactin released from the wild-type strain was defined as 100%. The data corresponds to mean values from three independent replicates. The bars indicate standard deviations. Asterisks indicate statistically significant differences ($p < 0.01$) according to two-tailed Student's *t*-tests. doi:10.1371/journal.pone.0108642.g002

manufacture of the standard, with monitoring at 250 nm. Peaks were identified using HPLC-grade enterobactin standards (EMC Microcollections GmbH, Tübingen, Germany). The amount of enterobactin exported was calculated from peak areas and normalized to that of the wild-type using HITACHI Model D-2000 Elite HPLC System Manager software.

Results

TolC is required for enterobactin export

Bleuel *et al.* have shown that TolC is involved in the efflux of enterobactin across the outer membrane of *E. coli* [30]. We performed HPLC analysis to confirm whether a strain with a deletion of *tolC* gene differs from the wild-type in its ability to release enterobactin. The *E. coli* wild-type and Δ tolC strains were grown for 13 h and then enterobactin was extracted from

supernatants of these cultures for RP HPLC analysis. Deletion of *tolC* resulted in a decrease in the export of enterobactin compared with the wild-type strain (Figure 1A). When the peak area of enterobactin released from the wild-type strain was defined as 100%, enterobactin release from Δ tolC was decreased to 47% (Figure 1B).

Effect of deletion of individual TolC-dependent drug efflux genes and *entS* on enterobactin release

To investigate the role of the TolC-dependent RND-type drug efflux systems on the release of enterobactin from *E. coli*, we performed RP HPLC analysis of the culture supernatants from cultures of *E. coli* MG1655 mutants containing single deletions of the *acrB*, *acrD*, *mdtABC*, *acrEF*, and *mdtEF* genes. We also investigated the effect of the *entS* deletion to confirm that it is

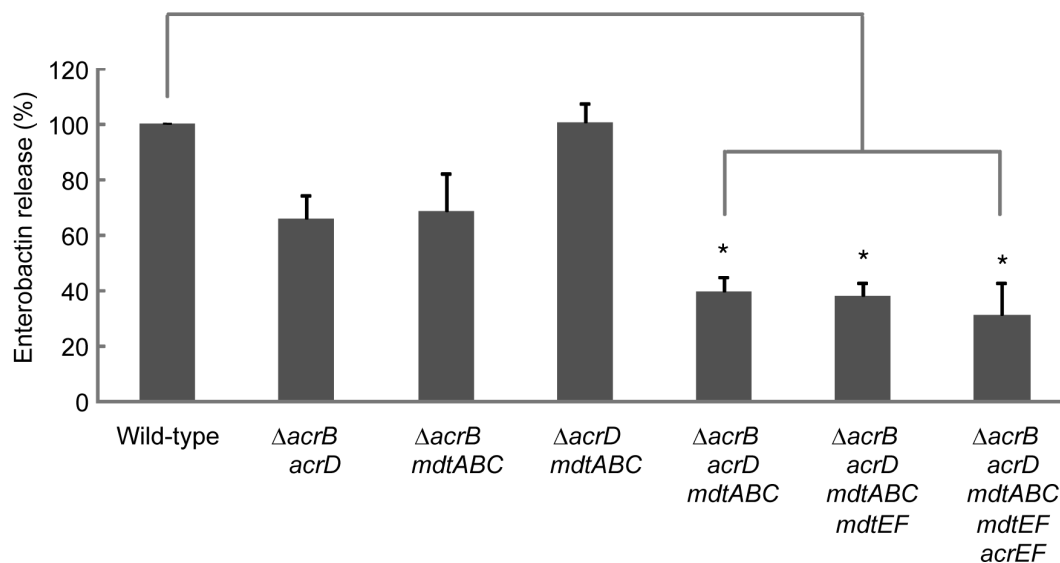


Figure 3. Requirement of AcrB, AcrD, and MdtABC drug efflux systems for enterobactin export. Enterobactin was prepared from the supernatants of cultures of each multiple RND transporter mutant and analyzed by RP HPLC. The amount of enterobactin exported by each strain, calculated using each peak area, is shown. The amount of enterobactin released from the wild-type strain was defined as 100%. The data correspond to mean values from three independent replicates. The bars indicate standard deviations. Asterisks indicate statistically significant differences ($p < 0.01$) according to the two-tailed Student's *t*-tests. doi:10.1371/journal.pone.0108642.g003

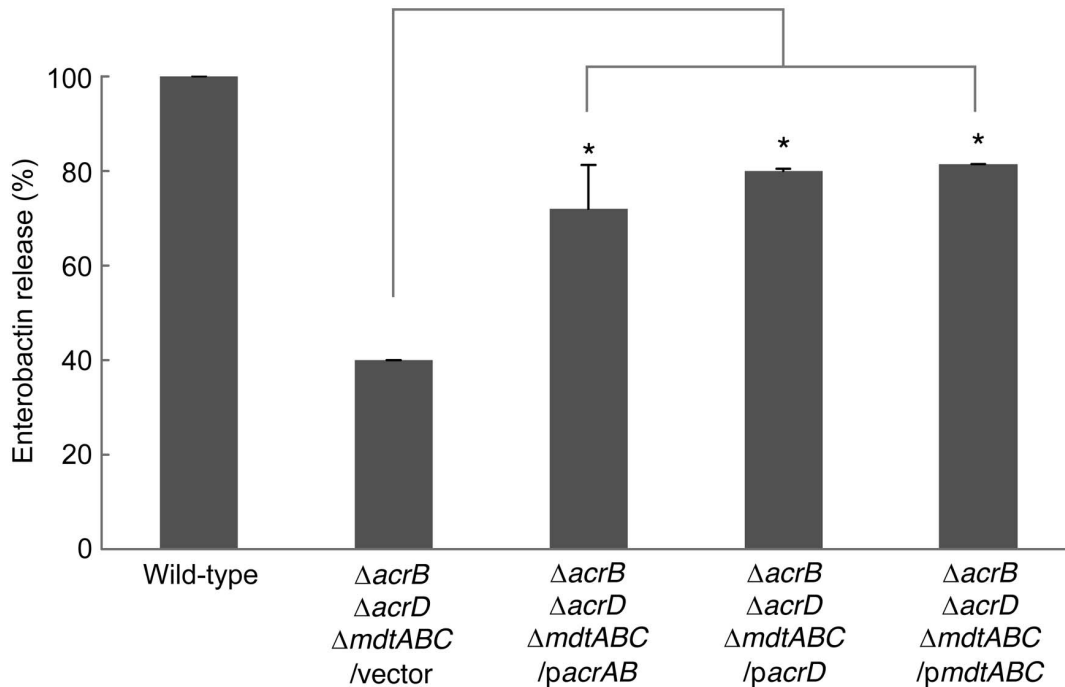


Figure 4. Complementation of enterobactin release from the $\Delta acrB$ $acrD$ $mdtABC$ mutant using plasmids carrying *acrB*, *acrD*, or *mdtABC* genes. The amounts of enterobactin exported by deletion mutants, calculated using peak areas, are shown. The amount of enterobactin released from the wild-type strain harboring an empty vector was defined as 100%. The data corresponds to mean values from three independent replicates. The bars indicate standard deviations. Asterisks indicate statistically significant differences ($p < 0.01$) determined using the two-tailed Student's *t*-tests.

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involved in enterobactin export [28] Although the amounts of enterobactin released from $\Delta tolC$ and $\Delta entS$ were significantly lower than that from the wild-type strain, there was no difference among the $\Delta acrB$, $\Delta acrD$, $\Delta mdtABC$, $\Delta acrEF$, $\Delta mdtEF$, and wild-type strains (Figure 2). These results are in agreement with a previous report [30].

AcrB, AcrD, and MdtABC drug efflux systems are required for enterobactin export

Because individual deletions of the *acrB*, *acrD*, *mdtABC*, *acrEF*, or *mdtEF* gene did not affect enterobactin release from *E. coli*, we hypothesized that multiple efflux systems may be involved in enterobactin export. To investigate this possibility, we performed analyses using multiple deletion mutants. The growth rates of the double mutants $\Delta acrB$ $acrD$, $\Delta acrB$ $mdtABC$, and $\Delta acrD$ $mdtABC$ were almost the same as that of the wild-type strain. The mutants $\Delta acrB$ $acrD$ $mdtABC$, $\Delta acrB$ $acrD$ $mdtABC$ $mdtEF$, and $\Delta acrB$ $acrD$ $mdtABC$ $mdtEF$ $acrEF$ grew slightly slower than the wild-type for the initial 3 h after inoculation; however, they grew to the same level as the wild-type after 13 h, when the samples were collected to measure enterobactin release. The double mutants $\Delta acrB$ $acrD$ and $\Delta acrB$ $mdtABC$ exported enterobactin to levels 66% and 69% of the level exported by wild type, respectively (Figure 3). Furthermore, deletion of *acrB*, *acrD*, and *mdtABC* also significantly decreased enterobactin export, to only 40% of the level exported by wild type. In contrast, the double mutant $\Delta acrD$ $mdtABC$ did not alter the ability of the wild-type to export enterobactin. Stepwise deletion of the *mdtEF* and *acrEF* genes from the $\Delta acrB$ $acrD$ $mdtABC$ mutant did not affect its ability to excrete enterobactin (Figure 3). These data indicate that, among the five RND transporters of *E. coli*, only AcrB, AcrD, and MdtABC play a role in the excretion of enterobactin.

AcrB, AcrD, and MdtABC are involved in enterobactin export

To confirm that AcrB, AcrD, and MdtABC are involved in enterobactin export, we performed a complementation analysis. The *acrB*, *acrD* and *mdtABC* genes were each cloned into the vector pTrc99A, and the resulting plasmids were used to investigate their ability to complement the enterobactin export defective phenotype of the $\Delta acrB$ $acrD$ $mdtABC$ mutant. All three plasmids increased enterobactin excretion from the $\Delta acrB$ $acrD$ $mdtABC$ mutant. The amounts of enterobactin released from the strains complemented with these plasmids were 72%, 80%, and 81% of the level released by the wild-type strain, respectively, which is nearly two-fold greater than that of the $\Delta acrB$ $acrD$ $mdtABC$ mutant harboring an empty control vector (Figure 4). These data indicates that AcrB, AcrD, and MdtABC are involved in enterobactin export in *E. coli*.

Discussion

In this study, we examined the involvement of the RND transporters in enterobactin export and the results indicate that AcrB, AcrD, and MdtABC are required for the secretion of enterobactin. The iron-chelating compound enterobactin is synthesized in the cytoplasm and exported to the growth medium to acquire iron. The MF-type transporter EntS was previously shown to be involved in enterobactin transport across the cytoplasmic membrane [28]. It has been believed that transporters in addition to EntS are involved in enterobactin export. Outer membrane channel TolC was proposed as a candidate for enterobactin export because of its wide substrate specificity [39] and ability to cooperate with multiple systems, including MF- and RND-type transporters [40,41]. Another study demonstrated that

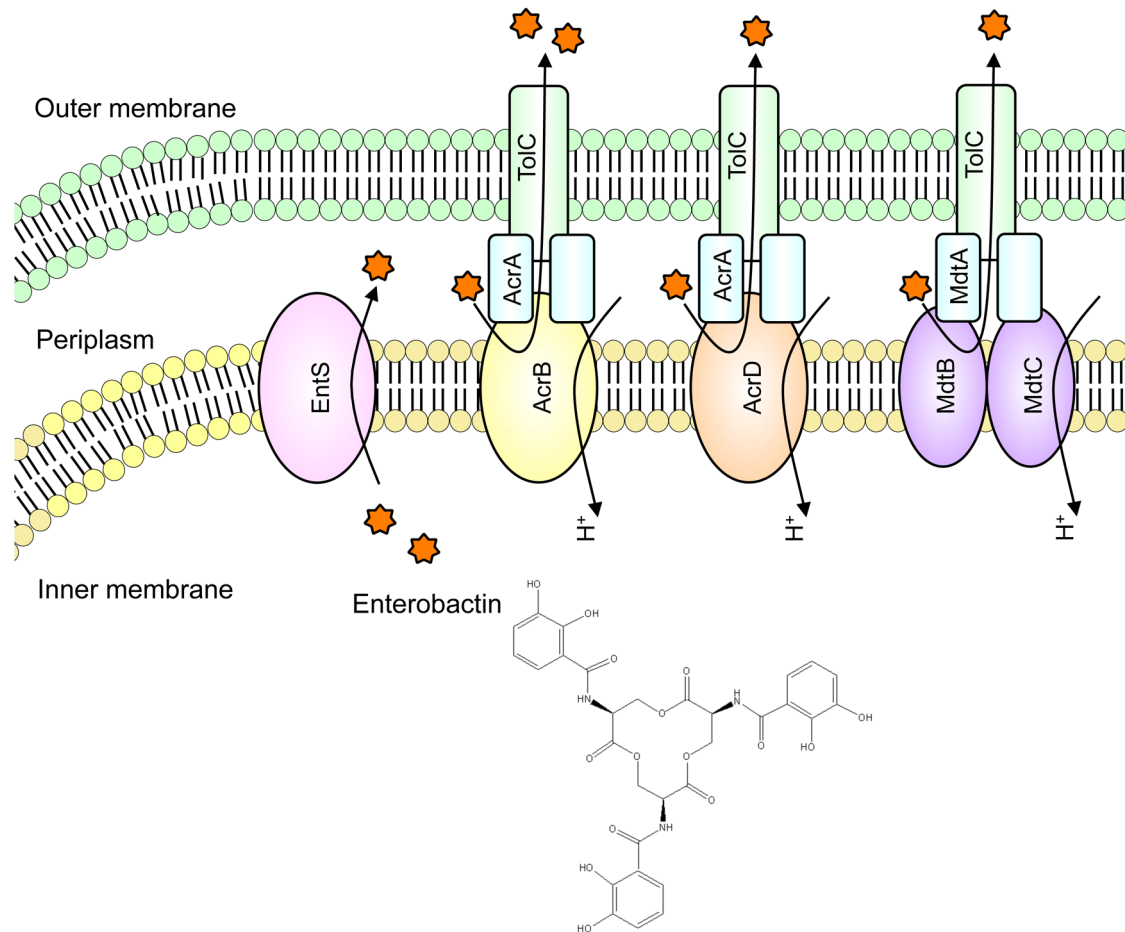


Figure 5. Proposed model of enterobactin export in *E. coli*. Enterobactin is synthesized in the cytoplasm and exported to the periplasm by EntS. The RND transporters AcrB, AcrD, and MdtABC capture enterobactin in the periplasm and then export it to the growth medium throughout the outer membrane channel TolC.
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deletion of *tolC* leads to the abolishment of enterobactin export and suggested that TolC is involved in enterobactin export from the periplasm to the growth medium [30]. In our study, deletion of *tolC* resulted in a decrease in enterobactin release, but it did not result in a complete loss of the ability to excrete enterobactin; the *tolC* deletion mutant could still export enterobactin to some extent. We believed that this resulted, in part, from differences in strain background. We used wild-type MG1655 as the background strain, but Δfur was used in a previous study [30]. Fur encodes the global regulator of iron homeostasis and deletion of *fur* results in constitutive production of enterobactin [42]. Because deletion of *fur* can affect enterobactin production and export, a *tolC* deletion may show a greater effect in this genetic background.

Individual deletions of *acrB*, *acrD*, and *mdtABC* did not affect the ability of *E. coli* cells to excrete enterobactin, whereas a triple deletion of these genes resulted in a significant decrease in enterobactin export. These three genes may not be unique in their ability to mediate enterobactin excretion, but AcrB, AcrD, and MdtABC coordinately play a role in enterobactin export from the periplasm to the growth medium in *E. coli* (Figure 5). Considering the results that double mutants $\Delta acrB\ acrD$ and $\Delta acrB\ mdtABC$ showed a decrease in enterobactin export, whereas $\Delta acrD\ mdtABC$ did not change its ability to export enterobactin, we speculate that AcrB plays a more pivotal role than AcrD and MdtABC.

The AcrAB–TolC system is constitutively expressed, but the expression levels of *acrD* and *mdtABC* are quite low under normal conditions [43]. We speculate the *acrD* and *mdtABC* genes may be induced when bacteria require iron to survive. Recently, our study on the identification of negative regulators for *acrD* and *mdtABC* revealed that the expression levels of these genes were affected by Fur (data not shown). A recent study by Ruiz and Levy also showed that inactivation of the enterobactin biosynthetic genes affects the expression level of *acrAB* [44]. These results suggest that multidrug transporters contribute to bacterial iron homeostasis, in addition to their role in multidrug resistance.

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Author Contributions

Conceived and designed the experiments: TH KN. Performed the experiments: TH KN. Analyzed the data: TH KN. Contributed reagents/materials/analysis tools: KN. Contributed to the writing of the manuscript: TH KN.

References

- Nikaido H (2011) Structure and mechanism of RND-type multidrug efflux pumps. *Adv Enzymol Relat Areas Mol Biol* 77: 1–60.
- Nikaido H (1996) Multidrug efflux pumps of gram-negative bacteria. *J Bacteriol* 178: 5853–5859.
- Murakami S, Nakashima R, Yamashita E, Matsumoto T, Yamaguchi A (2006) Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. *Nature* 443: 173–179.
- Nakashima R, Sakurai K, Yamasaki S, Nishino K, Yamaguchi A (2011) Structures of the multidrug exporter AcrB reveal a proximal multisite drug-binding pocket. *Nature* 480: 565–569.
- Ma D, Cook DN, Hearst JE, Nikaido H (1994) Efflux pumps and drug resistance in gram-negative bacteria. *Trends Microbiol* 2: 489–493.
- Nikaido H, Zgurskaya HI (2001) AcrAB and related multidrug efflux pumps of *Escherichia coli*. *J Mol Microbiol Biotechnol* 3: 215–218.
- Nishino K, Yamada J, Hirakawa H, Hirata T, Yamaguchi A (2003) Roles of TolC-dependent multidrug transporters of *Escherichia coli* in resistance to β -lactams. *Antimicrob Agents Chemother* 47: 3030–3033.
- Bina JE, Mekalanos JJ (2001) *Vibrio cholerae* tolC is required for bile resistance and colonization. *Infect Immun* 69: 4681–4685.
- Rosenberg EY, Bertenthal D, Nilles ML, Bertrand KP, Nikaido H (2003) Bile salts and fatty acids induce the expression of *Escherichia coli* AcrAB multidrug efflux pump through their interaction with Rob regulatory protein. *Mol Microbiol* 48: 1609–1619.
- Thanassi DG, Cheng LW, Nikaido H (1997) Active efflux of bile salts by *Escherichia coli*. *J Bacteriol* 179: 2512–2518.
- Aono R, Tsukagoshi N, Yamamoto M (1998) Involvement of outer membrane protein TolC, a possible member of the mar-sox regulon, in maintenance and improvement of organic solvent tolerance of *Escherichia coli* K-12. *J Bacteriol* 180: 938–944.
- Gilson L, Mahanty HK, Kolter R (1990) Genetic analysis of an MDR-like export system: the secretion of colicin V. *EMBO J* 9: 3875–3884.
- Guo X, Harrison RW, Tai PC (2006) Nucleotide-dependent dimerization of the C-terminal domain of the ABC transporter CvaB in colicin V secretion. *J Bacteriol* 188: 2383–2391.
- Delgado MA, Vincent PA, Farias RN, Salomon RA (2005) YojI of *Escherichia coli* functions as a microcin J25 efflux pump. *J Bacteriol* 187: 3465–3470.
- Vakharia H, German GJ, Misra R (2001) Isolation and characterization of *Escherichia coli* tolC mutants defective in secreting enzymatically active alpha-hemolysin. *J Bacteriol* 183: 6908–6916.
- Wandersman C, Delepelaire P (1990) TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. *Proc Natl Acad Sci U S A* 87: 4776–4780.
- Fralick JA (1996) Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. *J Bacteriol* 178: 5803–5805.
- Griffiths E (1991) Iron and bacterial virulence—a brief overview. *Biol Met* 4: 7–13.
- O'Brien IG, Gibson F (1970) The structure of enterochelin and related 2,3-dihydroxy-N-benzoylserine conjugates from *Escherichia coli*. *Biochim Biophys Acta* 215: 393–402.
- Pollack JR, Neilands JB (1970) Enterobactin, an iron transport compound from *Salmonella typhimurium*. *Biochem Biophys Res Commun* 38: 989–992.
- Liu J, Quinn N, Berchtold GA, Walsh CT (1990) Overexpression, purification, and characterization of isochorismate synthase (EntC), the first enzyme involved in the biosynthesis of enterobactin from chorismate. *Biochemistry* 29: 1417–1425.
- Buchanan SK, Smith BS, Venkatramani L, Xia D, Esser L, et al. (1999) Crystal structure of the outer membrane active transporter FepA from *Escherichia coli*. *Nat Struct Biol* 6: 56–63.
- Rutz JM, Liu J, Lyons JA, Goranson J, Armstrong SK, et al. (1992) Formation of a gated channel by a ligand-specific transport protein in the bacterial outer membrane. *Science* 258: 471–475.
- Bryce GF, Brot N (1972) Studies on the enzymatic synthesis of the cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine in *Escherichia coli*. *Biochemistry* 11: 1708–1715.
- Langman L, Young IG, Frost GE, Rosenberg H, Gibson F (1972) Enterochelin system of iron transport in *Escherichia coli*: mutations affecting ferric-enterochelin esterase. *J Bacteriol* 112: 1142–1149.
- Greenwood KT, Luke RK (1978) Enzymatic hydrolysis of enterochelin and its iron complex in *Escherichia coli* K-12. Properties of enterochelin esterase. *Biochim Biophys Acta* 525: 209–218.
- Brickman TJ, McIntosh MA (1992) Overexpression and purification of ferric enterobactin esterase from *Escherichia coli*. Demonstration of enzymatic hydrolysis of enterobactin and its iron complex. *J Biol Chem* 267: 12350–12355.
- Furrer JL, Sanders DN, Hook-Barnard IG, McIntosh MA (2002) Export of the siderophore enterobactin in *Escherichia coli*: involvement of a 43 kDa membrane exporter. *Mol Microbiol* 44: 1225–1234.
- Crouch ML, Castor M, Karlinsey JE, Kalhorn T, Fang FC (2008) Biosynthesis and IroC-dependent export of the siderophore salmochelin are essential for virulence of *Salmonella enterica* serovar Typhimurium. *Mol Microbiol* 67: 971–983.
- Bleuel C, Grosse C, Taudte N, Scherer J, Wesenberg D, et al. (2005) TolC is involved in enterobactin efflux across the outer membrane of *Escherichia coli*. *J Bacteriol* 187: 6701–6707.
- Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, et al. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* 277: 1453–1462.
- Winkelmann G, Cansier A, Beck W, Jung G (1994) HPLC separation of enterobactin and linear 2,3-dihydroxybenzoylserine derivatives: a study on mutants of *Escherichia coli* defective in regulation (*fur*), esterase (*fes*) and transport (*fepA*). *Biometals* 7: 149–154.
- Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* 97: 6640–6645.
- Nishino K, Senda Y, Yamaguchi A (2008) The AraC-family regulator GadX enhances multidrug resistance in *Escherichia coli* K-12. *Science* 277: 1453–1462.
- Nishino K, Senda Y, Hayashi-Nishino M, Yamaguchi A (2009) Role of the AraC-XylS family regulator YdeO in multi-drug resistance of *Escherichia coli*. *J Antibiot* 62: 251–257.
- Nishino K, Yamasaki S, Hayashi-Nishino M, Yamaguchi A (2011) Effect of overexpression of small non-coding DsrA RNA on multidrug efflux in *Escherichia coli*. *J Antimicrob Chemother* 66: 291–296.
- Nishino K, Yamasaki S, Hayashi-Nishino M, Yamaguchi A (2010) Effect of NlpE overproduction on multidrug resistance in *Escherichia coli*. *Antimicrob Agents Chemother* 54: 2239–2243.
- Link AJ, Phillips D, Church GM (1997) Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol* 179: 6228–6237.
- Koronakis V (2003) TolC—the bacterial exit duct for proteins and drugs. *FEBS Lett* 555: 66–71.
- Gerken H, Misra R (2004) Genetic evidence for functional interactions between TolC and AcrA proteins of a major antibiotic efflux pump of *Escherichia coli*. *Mol Microbiol* 54: 620–631.
- Zgurskaya HI, Nikaido H (1999) Bypassing the periplasm: reconstitution of the AcrAB multidrug efflux pump of *Escherichia coli*. *Proc Natl Acad Sci U S A* 96: 7190–7195.
- Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* 160: 47–56.
- Hirakawa H, Inazumi Y, Masaki T, Hirata T, Yamaguchi A (2005) Indole induces the expression of multidrug exporter genes in *Escherichia coli*. *Mol Microbiol* 55: 1113–1126.
- Ruiz C, Levy SB (2014) Regulation of *acrAB* expression by cellular metabolites in *Escherichia coli*. *J Antimicrob Chemother* 69: 390–399.