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 ΔacrB acrD mdtABC mutant. These results indicate that AcrB, AcrD, and MdtABC are required for the secretion of enterobactin.

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.
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 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 ΔtolC, ΔacrB, ΔacrD, ΔmdtABC, ΔmdtEF, ΔacrB ΔacrD, ΔacrB ΔmdtABC, and ΔacrB ΔacrD ΔmdtABC were constructed as previously described [34–37]. The oligonucleotide primers acrE-P1 (TTGGTAAATCAAGCGGTGTGGTTTGGTTTGGAGGAAATAGTAGTGAGCGTGAGCTGTC) and acrF-P2 (AAATATAGATGCGAGCAGCCTTATGAGGCTTTATATATACGATGATAGTATATACCTTCTTAG) were used to construct the ΔacrEF mutant. The kanamycin resistance gene aph, flanked by FLP 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

| Strain or Plasmid | Original name | Characteristics | Source or references |
|-------------------|---------------|-----------------|---------------------|
| Wild-type         | MG1655        | Escherichia coli wild type | 31                   |
| ΔacrB             | NKE96         | ΔacrB           | 36                   |
| ΔacrD             | NKE94         | ΔacrD::Cm<sup>+</sup> | 37                   |
| ΔmdtABC           | NKE133        | ΔmdtABC::Km<sup>+</sup> | 37                   |
| ΔacrEF            | NKE129        | ΔacrEF::Km<sup>+</sup> | This study           |
| ΔmdtEF            | NKE138        | ΔmdtEF::Km<sup>+</sup> | 34                   |
| ΔtolC             | NKE95         | ΔtolC::Cm<sup>+</sup> | 35                   |
| ΔentS             | NKE869        | ΔentS           | This study           |
| ΔacrB ΔacrD       | NKE126        | ΔacrB ΔacrD     | 37                   |
| ΔacrB mdtABC      | NKE141        | ΔacrB ΔmdtABC::Km<sup>+</sup> | 37                   |
| ΔacrD mdtABC      | NKE1288       | ΔacrD ΔmdtABC::Cm<sup>+</sup> ΔacrD::Cm<sup>+</sup> | This study           |
| ΔacrB ΔacrD mdtABC | NKE1317      | ΔacrB ΔacrD ΔmdtABC | 37                   |
| ΔacrB ΔacrD ΔmdtABC ΔmdtEF | NKE1327 | ΔacrB ΔacrD ΔmdtABC ΔmdtEF | This study           |
| ΔacrB ΔacrD ΔmdtABC ΔmdtEF ΔacrEF | NKE1329 | ΔacrB ΔacrD ΔmdtABC ΔmdtEF ΔacrEF::Km<sup>+</sup> | This study           |
| ΔacrB ΔacrD ΔmdtABC::vector | NKE1575 | ΔacrB ΔacrD ΔmdtABC::FlpR::Km<sup>+</sup> | This study           |
| ΔacrB ΔacrD ΔmdtABC::pacrAB | NKE1576 | ΔacrB ΔacrD ΔmdtABC::pacrAB | This study           |
| ΔacrB ΔacrD ΔmdtABC::pacrD | NKE1578 | ΔacrB ΔacrD ΔmdtABC::pacrD | This study           |
| ΔacrB ΔacrD ΔmdtABC::pmdtABC | NKE1583 | ΔacrB ΔacrD ΔmdtABC::pmdtABC | This study           |

| Vector            | rep<sub>psK</sub> Ap<sup>+</sup> FRT Km<sup>+</sup> FRT | 33                   |
|                   | rep<sub>psK</sub> Ap<sup>+</sup> Km<sup>+</sup> cia57/P<sub>F</sub>flp | 33                   |
| pTrc99A           | Vector, Ap<sup>+</sup> | Amerham Pharmacia Biotech |

Plasmids

pacrAB genes cloned into pTrc99A, Ap<sup>+</sup> | This study |

pacrD genes cloned into pTrc99A, Ap<sup>+</sup> | This study |

pmdtABC genes cloned into pTrc99A, Ap<sup>+</sup> | This study |

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Table 1. Strains and plasmids used in this study.
were verified by PCR using the primers acrE-F (GTTAAA-TAAATATATATATATTACCTA) and acrF-R (CGTGAGCAGCCCGCAGCAATGCGGTGA), and K-1 (CAGTCTAGCCGAATAGCGCCT) and K-2 (CGGTGCCTAGTAATGACTG). To construct ΔacrD mdtABC, ΔacrB acrD mdtABC mdtEF, and ΔacrB 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 ΔentS mutant, precise in-frame deletions were generated using crossover PCR. The oligonucleotide primers entS-No (CGCGGATCCAAAGGCAACAATTCAATGAGGC) and entS-Ni (CACGCAATAACCTTCACTCAGTCCAAATTTATAACCATTACAATGCCTTGCCATC), plus entS-Gi (GTTATAAATTTGGAGTGTGAAGGTTATTGCGTGTAATGCTTAAAACAGCGCCTTAAGCC), and entS-Co (CGCGTCGACGACGACAAAACGTGGCATGTATTTGCCTAGTTAAACACGCCCTTAAGCC), were used. Then the fragment containing the deletion was cloned into the BamHI 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 pHSGacrAB and pHSGacrD into vector pTrc99A using BamHI and HindIII, and HindIII and SacI, respectively [7]. PCR with PrimeSTAR GXL DNA polymerase (TaKaRa Bio Inc., Otsu, Japan) and the primers mdtABC-EcoRI (CGCCGAATTCAGAAATCTTTTTAGAGAA) and mdtABC-KpnI (CGCCGATACCTTACTGTTATCCGTGGTTTTTTTTT) 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 10⁸ cells of each strain. Supernatants of 13 h cultures were acidified with 50 μl 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 μl 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, 4.6×250 mm, 5 μm: 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 μl of each supernatant extraction was injected and separated as described by the
manufacture of the standard, with monitoring at 250 nm. Peaks were identified using HPLC-grade enterobactin standards (EMC Microcollections GmbH, Tubingen, 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 ΔtolC 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 ΔtolC 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 2. RP HPLC analysis of enterobactin released from deletion mutants of the RND-type efflux system genes.](image)

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.

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![Figure 3. Requirement of AcrB, AcrD, and MdtABC drug efflux systems for enterobactin export.](image)

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.

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involved in enterobactin export [28] Although the amounts of enterobactin released from \( \text{D}\acrB \) and \( \text{D}\entS \) were significantly lower than that from the wild-type strain, there was no difference among the \( \text{D}\acrB \), \( \text{D}\acrD \), \( \text{D}\mdtABC \), \( \text{D}\acrEF \), \( \text{D}\mdtEF \), 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 \( \text{acrB} \), \( \text{acrD} \), \( \text{mdtABC} \), \( \text{acrEF} \), or \( \text{mdtEF} \) gene did not affect enterobactin release from \( \text{E. coli} \), 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 \( \text{D}\acrB\acrD \), \( \text{D}\acrB\mdtABC \), and \( \text{D}\acrD\mdtABC \) were almost the same as that of the wild-type strain. The mutants \( \text{D}\acrB\acrD\mdtABC \), \( \text{D}\acrB\acrD\mdtABC\mdtEF \), and \( \text{D}\acrB\acrD\mdtABC\mdtEF\acrEF \) 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 \( \text{D}\acrB\acrD \) and \( \text{D}\acrB\mdtABC \) exported enterobactin to levels 66% and 69% of the level exported by wild type, respectively (Figure 3). Furthermore, deletion of \( \text{acrB} \), \( \text{acrD} \), and \( \text{mdtABC} \) also significantly decreased enterobactin export, to only 40% of the level exported by wild type. In contrast, the double mutant \( \text{D}\acrD\mdtABC \) did not alter the ability of the wild-type to export enterobactin. Stepwise deletion of the \( \text{mdtEF} \) and \( \text{acrEF} \) genes from the \( \text{D}\acrB\acrD\mdtABC \) mutant did not affect its ability to excrete enterobactin (Figure 3). These data indicate that, among the five RND transporters of \( \text{E. coli} \), only AcrB, AcrD, and MdtABC play a role in the excretion of enterobactin.

**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. The \( \text{acrB} \), \( \text{acrD} \), and \( \text{mdtABC} \) genes were each cloned into the vector pTc99A, and the resulting plasmids were used to investigate their ability to complement the enterobactin export defective phenotype of the \( \text{D}\acrB\acrD\mdtABC \) mutant. All three plasmids increased enterobactin excretion from the \( \text{D}\acrB\acrD\mdtABC \) 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 \( \text{D}\acrB\acrD\mdtABC\mdtEF \) mutant harboring an empty control vector (Figure 4). These data indicates that AcrB, AcrD, and MdtABC are involved in enterobactin export in \( \text{E. coli} \).

Figure 4. Complementation of enterobactin release from the \( \text{D}\acrB\acrD\mdtABC \) mutant using plasmids carrying \( \text{acrB} \), \( \text{acrD} \), or \( \text{mdtABC} \) 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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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 \( \text{acrB} \), \( \text{acrD} \) and \( \text{mdtABC} \) 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 \( \text{D}\acrB\acrD\mdtABC \) mutant. All three plasmids increased enterobactin excretion from the \( \text{D}\acrB\acrD\mdtABC \) 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 \( \text{D}\acrB\acrD\mdtABC\mdtEF \) mutant harboring an empty control vector (Figure 4). These data indicates that AcrB, AcrD, and MdtABC are involved in enterobactin export in \( \text{E. coli} \).
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 _D fur_ 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 transport from the periplasm to the growth medium in _E. coli_ (Figure 5). Considering the results that double mutants _ΔacrB acrD_ and _ΔacrB mdtABC_ showed a decrease in enterobactin export, whereas _ΔacrD 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.
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