Molecular Characterization of a Chromosomal Determinant Conferring Resistance to Zinc and Cobalt Ions in Staphylococcus aureus

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A DNA fragment conferring resistance to zinc and cobalt ions was isolated from a genomic DNA library of Staphylococcus aureus RN450. The DNA sequence analysis revealed two consecutive open reading frames, designated zntR and zntA. The predicted ZntR and ZntA showed significant homology to members of ArsR and cation diffusion families, respectively. A mutant strain containing the null allele of zntA was more sensitive to zinc and cobalt ions than was the parent strain. The metal-sensitive phenotype of the mutant was complemented by a 2.9-kb DNA fragment containing zntR and zntA. An S. aureus strain harboring multiple copies of zntR and zntA showed an increased resistance to zinc. The resistance to zinc in the wild-type strain was inducible. Transcriptional analysis indicated that zntR and zntA genes were cotranscribed. The zinc uptake studies suggested that the zntA product was involved in the export of zinc ions out of cells.

The trace heavy metal ions such as cobalt, zinc, copper, and nickel play important roles in bacteria. They regulate a wide array of metabolic functions as coenzymes or cofactors, as catalysts or Lewis acid in enzymes, and as structural stabilizers of enzymes and DNA-binding proteins (9, 18). However, these trace heavy metal ions are toxic in excess of normal physiological levels (28). Increasing environmental concentrations of these heavy metals pose a challenge to bacteria. Therefore, bacteria have evolved mechanisms to regulate the influx and efflux processes to maintain the relatively steady intracellular level of the heavy metal ions. Different molecular mechanisms have been reported that are responsible for resistance to various trace heavy metal ions in bacteria (2, 8, 13, 18, 22, 23, 27). The molecular mechanisms involve a number of proteins, such as ion transporters, reductases, glutathione-related cadystins and cysteine-rich metallothioneins, and low-molecular-weight cysteine-rich metal ligands (27). These protein molecules either export the metal ions out of cells or detoxify or sequester them so that the cells can grow in an environment containing high levels of toxic metals. However, there is no common mechanism of resistance to all heavy metal ions. In bacteria, the genes encoding resistance to heavy metals are located either on the bacterial chromosome, on the plasmids, or on both (18, 27).

Staphylococcus aureus is a common human pathogen associated with a number of diseases. Understanding of metal resistance in staphylococci has progressed rapidly in the past 10 years, with well-established cadmium, mercury, antimony, and arsenic resistance systems encoded by plasmids (20, 25, 27). However, staphylococcal strains without plasmids show resistance in staphylococci has progressed rapidly in the past 10 years, with well-established cadmium, mercury, antimony, and arsenic resistance systems encoded by plasmids (20, 25, 27). In bacteria, the genes encoding resistance to heavy metals are located either on the bacterial chromosome, on the plasmids, or on both (18, 27).

Staphylococcus aureus is a common human pathogen associated with a number of diseases. Understanding of metal resistance in staphylococci has progressed rapidly in the past 10 years, with well-established cadmium, mercury, antimony, and arsenic resistance systems encoded by plasmids (20, 25, 27). However, staphylococcal strains without plasmids show resistance to heavy metal ions, such as zinc and cobalt. This implies that a plasmid-independent chromosomal determinant might encode resistance to heavy metals such as zinc and cobalt. Although operons encoding cobalt, zinc, and cadmium in Alcaligenes eutrophus (17) and zinc in Escherichia coli (2) have been investigated, relatively little is known about the transport of and resistance mechanisms to zinc and cobalt ions in S. aureus. Here we report the cloning, sequencing, and genetic analysis of a determinant located on the bacterial chromosome that codes for zinc and cobalt resistance in S. aureus.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. S. aureus strains were grown on Luria-Bertani (LB) agar or broth at 37°C with shaking (200 rpm). When necessary for selection, ampicillin (50 μg/ml), kanamycin (30 μg/ml for E. coli, 500 μg/ml for S. aureus), and chloramphenicol (20 μg/ml for E. coli, 10 μg/ml for S. aureus) were added to the media. When required, an appropriate volume of filter-sterilized 0.5 M stock solutions of ZnCl₂ or CoCl₂ was added to TSB. The biomass in liquid cultures was estimated from the optical density at 580 nm (OD₅₈₀) with a DU-64 Beckman spectrophotometer. Cell yield was determined from a calibration curve relating OD₅₈₀ to cell dry weight.

DNA manipulation and sequencing. All standard methods of DNA manipulation were performed according to the protocols of Novick (19) and Sambrook et al. (26). Genomic DNA of S. aureus was isolated by using DNAzol kits (Molecular Research Center, Inc., Cincinnati, Ohio). Plasmid was purified with the Qiagen plasmid minipreparation kit (Qiagen, Inc., Chatsworth, Calif.). PCR-amplified products and DNA fragments from agarose gels were purified withQiagulick gel extraction kits. DNA probes were labeled by using the Repliprint DNA labeling system (Amersham Life Science, Arlington Heights, Ill.). All DNA restriction and modification enzymes were obtained from Promega (Madison, Wis.) and used according to the manufacturer’s instructions. DNA sequences were determined with an ABI Prism 310 genetic analyzer system (Perkin-Elmer, Foster City, Calif.). Two pairs of oligonucleotide primers were used for PCR amplification: PCA1 and PCA2 (5′-TAAAGGCCGCGCAGACTTCAAC-3′ and 5′-CTTGTTGTGTTTGGCACAATGTG-3′) and CAF1 (5′-TTGATGATCCATGCACTGCACT-3′ and 5′-GACCAAACAAA GTGCCATAAAGAC-3′). DNA sequences were analyzed by the MacVector (version 5.0) program, and multiple protein alignments were performed by the ClustalW program (29).

Construction of the zntA mutant and complementation. The 2.9-kb EcoRI fragment containing zntR and zntA was cloned into vector pTZ18R. The resulting plasmid pTZ18R-ZC (5.8 kb) was digested with SstI and Smal to remove the AccI site from the multicloning site of the vector and then religated after end filling with Klenow DNA polymerase. The resulting plasmid was digested with AccI to insert a 1.5-kb kanamycin cassette from pOS7kan (11). The EcoRI fragment containing the kanamycin cassette within zntA was then subcloned into the pBT2 shuttle vector that contained a temperature-sensitive staphylococcal origin of replication (4). The resulting plasmid pBT2-ZCK was electroporated into competent S. aureus RN4220 cells. Selection for double-crossover events with the chromosome of S. aureus was carried out at 43°C as described previously (3, 4). One representative mutant was analyzed by Southern blotting in order to exclude possible recombination adjacent to the insertion sites or a single crossover event by using the 2.9-kb EcoRI fragment as a probe. The exact insertion site of the kanamycin cassette on the chromosome was confirmed by nucleotide sequencing of the PCR-amplified product with the primers PCA1 and PCA2.
complement the mutation in trans, the 2.9-kb EcoRI fragment containing zntR and zntA was cloned into the pCU1 shuttle vector (1). The resulting plasmid, pCU1-ZC, was electroporated into the mutant strain RN-MZ.

Analysis of zinc ion accumulation. Zinc concentration was measured as described by Headard et al. (2). Cultures grown overnight were transferred to 40 ml of fresh TSB to give an OD$_{680}$ of approximately 0.1. When the optical density of cultures came close to 1.0, appropriate amounts of ZnCl$_2$ were added to the cultures to give various final concentrations. The cultures were then incubated for an additional 4 h. Then, 25-ml aliquots of the cultures described above were centrifuged at 3,000 $g$ for 15 min. Cell pellets were washed with 4 ml of TSB and then with 4 ml of 0.1 N HNO$_3$ to remove the surface-bound zinc ions. The intracellular concentrations of zinc were determined with an atomic absorption spectrophotometer (Thermo Jarrell Ash Corp., Franklin, Mass.) (2).

Induction of znt transcription. The wild-type S. aureus strains were grown under the following conditions to evaluate the induction of znt operon in the presence of zinc ions: (i) in TSB without zinc, (ii) in TSB without zinc to mid-log phase and then subcultured (1:100) into TSB containing 1.5 mM zinc, and (iii) in TSB containing 0.5 and 2 mM zinc to mid-log phase and then subcultured (1:100) in TSB containing 1.5 mM zinc. The cells were harvested after 6 h, and the total RNA was isolated with the TRI reagent kit (Molecular Research Center). Then, 10 $\mu$g of total RNA from each sample was electrophoresed on formaldehyde agarose gels (1.0%) and transferred to nitrocellulose membrane. Membranes were prehybridized for 8 to 12 h and then hybridized overnight with rose gels (1.0%) and subsequently autoradiographed.

Primer extension. The primer extension assay was performed using an oligonucleotide primer of 19 bases (5'-GTTAATCGCTAATAGCCTTTG-3') specific to the zntR coding region. The primer (10 ng) and total RNA (10 $\mu$g) from wild-type S. aureus were combined in 3 $\mu$l of water and boiled for 1 min followed by quick cooling in ice water. The primer extension reaction was carried out in a total volume of 10 $\mu$l containing 2 $\mu$l of 5 X AMV buffer; 2 $\mu$l of a 250- $\mu$M dATP, dTTP, and dGTP mix; 1 $\mu$l of [Y-32P]dCTP; 12 U of avian myeloblastosis virus (AMV) reverse transcriptase; and 2 U of RNasin. The mixture was incubated at 37°C for 30 min. Chase solution (3 $\mu$l) containing 25-mm concentrations of dGTP, dCTP, dTTP, and dATP was then added, and the mixture was incubated for an additional 30 min at 37°C. The reaction was stopped by the addition of an equal volume of 98% formamide, 0.3% xylene cyanol, and 0.3% bromophenol blue. Denatured samples (5 $\mu$l) were electrophoresed on denaturing polyacrylamide gels. A sequence ladder generated by using the same primer on a 2.9-kb EcoRI fragment was coelectrophoresed and used to determine the position of the transcription start site.

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to GenBank under accession number AF044951.

RESULTS AND DISCUSSION

Cloning and sequencing of S. aureus genes encoding zinc and cobalt resistance. The genomic library of S. aureus RN450 was constructed in the pCU1 shuttle vector (1). S. aureus RN450 was transformed with this pCU1-based S. aureus genomic library by electroporation (19). Approximately 2,000 S. aureus transformants were tested for zinc tolerance by replica plating on TSA medium containing 10 mM ZnCl$_2$. After incubating for 2 days at 37°C, the transformants that showed faster growth were further analyzed. The plasmids isolated from these clones were transformed into S. aureus. DNA from positive transformants yielded resistance to 10 mM ZnCl$_2$, suggesting that zinc resistance was due to a sequence contained on the plasmids. Further restriction analysis and zinc resistance studies indicated that the gene(s) encoding the zinc resistance were located within a 2.9-kb EcoRI fragment that was subsequently subcloned into the pTZ18R vector (16).

The nucleotide sequence of ~2.0 kb from the 2.9-kb EcoRI fragment revealed two consecutive open reading frames, designated zntR and zntA, of 318 and 978 bp and corresponding to 106 and 326 amino acid residues, respectively. The start codon of zntA is separated from the stop codon of zntR by a single base. The molecular masses of the putative proteins of zntR and zntA were predicted to be 11,987 and 36,230 Da, respectively. Putative Shine-Dalgarno sequences, GAAAGG and AGTGGG, were found upstream of the proposed initiation codons ATG of zntR and zntA, respectively. Also, possible −35 (TTGACA) and −10 (ATTAAA) sequences were identified upstream of zntR. An imperfect 8-2-8 bp inverted repeat (AATTATAG-AAAACAATATT) is located between the −10 region and the site of translation initiation, which represents a potential regulatory site (8). The typical −10 and −35 regions, however, are not present upstream of zntA, a finding consistent with cotranscription of zntR and zntA. Downstream of the stop codon of zntA, there is an inverted repeat, followed by a T-rich region which may function as a transcription termination structure. All of these features suggest that the zntR and zntA were organized in an operon on the S. aureus chromosome. The organization of the znt operon is similar to that of the ara operon, encoding a repressor and a structural gene for antimonite resistance in Staphylococcus xylosus (25).

Analysis of predicted amino acid sequences of ZntR and ZntA. The predicted ZntR sequence showed 32% identity of amino acid residues with SmtB of Synchococcus (8), 32% identity with CadC of S. aureus (32), 30% identity with ArsR of S. xylosus (25) and S. aureus (10), and 28% identity with ArsR of E. coli (6). All of these homologs are members of the ArsR family (6, 10). ZntR appeared to be a hydrophilic protein, suggesting a cytoplasmic location. The secondary structure and domain analysis of ZntR suggested that there was a consensus DNA-binding helix-turn-helix motif (7), but it lacks the conserved CXC sequence. A putative regulatory region with an

<table>
<thead>
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<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC8325-4</td>
<td>Laboratory strain cured of prophages</td>
<td>19</td>
</tr>
<tr>
<td>RN4220</td>
<td>NCTC8325-4, r−</td>
<td>This work</td>
</tr>
<tr>
<td>RN-MZ</td>
<td>RN4220, rzcB:kan (Kan')</td>
<td>This work</td>
</tr>
<tr>
<td>RN-CMZ</td>
<td>RN-MZ containing pCU1-ZC plasmid (Kan' Cm')</td>
<td>This work</td>
</tr>
<tr>
<td>RN-ZC</td>
<td>RN4220 containing pCU1-ZC plasmid (Cm')</td>
<td>This work</td>
</tr>
<tr>
<td>E. coli JM109</td>
<td>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (lac-proAB) F' (traD36 proAB+) lacI2Δ M15</td>
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<tr>
<td>pTZ18R-ZC</td>
<td>pTZ18R containing 2.9-kb EcoRI fragment conferring resistance to zinc and cobalt (Amp')</td>
<td>This work</td>
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<tr>
<td>pTZ18R-ZCK</td>
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<td>pCU1-ZC</td>
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<td>This work</td>
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<td>This work</td>
</tr>
<tr>
<td>pOSTKan</td>
<td>Containing kanamycin resistance cassette</td>
<td>11</td>
</tr>
</tbody>
</table>

*Table 1. Bacterial strains and plasmids used in this study*
inverted repeat was located upstream of zntR, which represented the potential binding site of a regulatory protein (8).

ZntA shared 38% identity with CzcD of A. eutrophus (17), 34% identity with zinc and cobalt resistance genes of yeast (5, 12) and 29% identity with ZnT1 of mice (22). The ZntA protein was predicted to have six transmembrane domains and a long hydrophilic C-terminal tail as reported for CzcD and other members of the cation diffusion family (23). ZntA had two histidine-rich regions, one at the C terminus (10 of 17 amino acids) and the other near the N terminus (8 of 12 amino acids). Similar histidine-rich regions have been reported for zinc and cobalt transporters which are thought to be the domains for binding the zinc ions (5, 12, 18). However, the CzcD of A. eutrophus, which functions as the sensor of a two-component regulatory system of cadmium, zinc, and cobalt, lacked any histidine-rich region (13). Also, in contrast to most bacterial transporters of heavy metal ions, the ZntA protein lacked the conserved CXXC motif for metal binding and had relatively low levels of cysteine residues (1.2%).

**FIG. 1. Construction of the zntA mutant.** First, a 2.9-kb EcoRI fragment containing zntR and zntA was cloned into pTZ18R (ΔSmal-SalI) to construct the pTZ18R-ZC plasmid (5.8 kb). A 1.5-kb fragment containing the kanamycin resistance cassette from pOSTkan was introduced into the AccI site within zntA by blunt-end ligation. From the resulting plasmid, pTZ18R-ZCK (7.3 kb), the EcoRI fragment was subcloned into the pBT2 shuttle vector to generate the pBT2-ZCK plasmid (11 kb). ERI, EcoRI; A, AccI; X, XbaI; P, PstI; Amp, ampicillin; Kan, kanamycin; Cat, chloramphenicol; ori E, E. coli origin of replication; ori S(ts), temperature-sensitive S. aureus origin of replication.
TABLE 2. MICs of zinc and cobalt ions for the different S. aureus strains used in this study

<table>
<thead>
<tr>
<th>S. aureus strain</th>
<th>Zn^{2+} (mM)</th>
<th>Co^{2+} (mM)</th>
</tr>
</thead>
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<tr>
<td>RN4220</td>
<td>5.0</td>
<td>3.0</td>
</tr>
<tr>
<td>RN-CMZ</td>
<td>5.0</td>
<td>3.0</td>
</tr>
<tr>
<td>RN-MZ</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>RN-ZC</td>
<td>&gt;10.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* MICs were determined by growing cells in 5 ml of TSB medium with appropriate concentrations of zinc and cobalt ions for 24 h.

A total of 4.3 kb in the parent to ~5.8 kb in the mutant RN-MZ (data not shown). The PCR analysis with primers PCA1 and PCA2 spanning the insertion site of the kanamycin cassette further proved that the size of the PCR product from the mutant strain was increased by ~1.5 kb compared to that of the wild type. Nucleotide sequence analyses of the PCR products from the mutant and the parent strains also confirmed the insertion of the kanamycin cassette at the AccI site of the zntA gene of the chromosome. As shown in Table 2, the MICs of zinc and cobalt for the mutant RN-MZ (zntA) were each 0.5 mM compared to 5 and 3 mM for the parent strain, respectively. Thus, the mutant RN-MZ (zntA) strain was sensitive to zinc and cobalt ions.

In order to determine whether the cloned DNA fragments (zntR and zntA) can complement in trans the zinc- and cobalt-sensitive phenotypes, plasmid pCU1-ZC was transformed into the mutant RN-MZ. As shown in Table 2, the MICs of zinc and cobalt for the mutant containing pCU1-ZC increased from 0.5 mM to 5 and 3 mM, respectively. Thus, the zinc- and cobalt-sensitive phenotypes of the mutant RN-MZ were fully complemented by the pCU1-ZC plasmid. The higher MIC of the RN-ZC compared to wild type (~2-fold) was due to the presence of the multiple copies of znt. However, no effect on the MICs of cadmium, nickel, copper, arsenic, and mercury was observed either in the parent or in the mutant strain. We also observed that the growth of the RN-MZ under normal physiological conditions was not affected and was similar to that of the wild type (data not shown). This suggested that the zntA was not essential for the growth of S. aureus under normal conditions.

Functional analysis of ZntA. The intracellular concentration of zinc was measured to determine whether ZntA is involved in the influx or efflux of zinc ions. S. aureus strains grown in TSB medium containing various concentrations of zinc were collected and washed, and digested (2) and were used to determine zinc concentration by atomic absorption spectrophotometry. As shown in Fig. 2, the mutant strain accumulated zinc ion to twice the level of the parent strain. In contrast, in the RN-ZC strain (containing multiple copies of znt) the intracellular zinc concentration was only one-half that of the parent strain. The accumulation of high concentrations of zinc in the zntA mutant is probably indicative of its inability to efflux zinc. The lower zinc concentration in RN-ZC further supports the involvement of ZntA in its transport. Again, the RN-ZC strain showed more-efficient efflux of zinc ions from cells compared to the parent strain due to an increased intracellular level of ZntA.

Analysis of znt transcript. A preliminary study was conducted to evaluate the effect of various concentrations of zinc on the growth of wild-type and mutant S. aureus strains. Northern blot analysis was performed to evaluate the expression of znt during growth of S. aureus strains in the presence or in the absence of zinc. Total RNA was isolated from cultures exposed to different concentrations of zinc and then separated by electrophoresis in 1% agarose. Northern blot analysis revealed the presence of only one transcription unit of ~1.4 kb when fragments containing a portion of zntR and zntA were used as probes (Fig. 3A). This size seemed to be in good agreement with that of the predicted 1.3-kb operon. It also implied that zntR and zntA were cotranscribed. Interestingly, as shown in Fig. 3A (lane 4), the zntA mutant also expressed a ~1.4-kb transcript size, which was almost equal to the transcript size of zntA in the wild-type strain (lane 5). We assumed that although the insertion inactivated zntA, it read through the kanamycin resistance gene and produced a ~1.4-kb transcript. When the kanamycin resistance cassette was used as a probe, Northern blotting revealed two hybridizing bands (~1.4 and ~1.2 kb) in the total mutant RNA (Fig. 3B). The ~1.2-kb unit is the transcription product of the kanamycin resistance cassette.

We observed that resistance to heavy metal ions was dependent not only on the gene copy number but also on the transcriptional product of the kanamycin resistance cassette.

FIG. 2. Zinc accumulation by RN4220, RN-ZC and the zntA mutant (RN-MZ) strains of S. aureus. The bacteria grown in TSB medium containing various concentrations of zinc were collected and washed, and the levels of intracellular zinc were determined by atomic absorption spectrometry. Symbols: ●, RN4220; ○, RN-MZ; ▲, RN-ZC.

![Zinc accumulation by S. aureus strains](image)

FIG. 3. Northern blot analysis of the zntR and zntA. Total RNA (10 μg) isolated from samples was separated electrophoretically and transferred by blotting onto a membrane. (A) Lanes: 1, S. aureus RN4220 grown with 2 mM zinc to mid-log phase and then diluted (1:100) in TSB medium containing 1.5 mM zinc; 2, S. aureus RN4220 grown with 0.5 mM zinc to mid-log phase and then diluted (1:100) in TSB medium containing 1.5 mM zinc; 3, S. aureus RN4220 grown without zinc to mid-log phase and then diluted (1:100) in TSB medium containing 1.5 mM zinc; 4, RN-MZ grown in TSB; 5, S. aureus RN4220 grown in TSB. The blot was probed with a radiolabeled DNA fragment encompassing zntR and zntA. The sizes of the rRNA are marked with arrows. (B) An RNA sample (10 μg) isolated from the mutant RN-MZ strain was separated electrophoretically and transferred by blotting onto a membrane. The blot was probed with a radiolabeled DNA fragment containing the kanamycin gene.
be due to a minor transcription start site in the (Fig. 4). The presence of a minor band in Fig. 4 (lane P) may inverted repeat that represented a potential regulatory site transcription unit more precisely, the 5\textsuperscript{initiation start (marked by an asterisk) is enlarged.}

reaction performed with the same primer. The sequencing encompassing the transcriptase (lane P). Lanes T, G, C, and A correspond to a dideoxy sequencing Total RNA from the parent strain RN4220 was hybridized with an oligonucleotide complementary to the mRNA of \textit{zntR} locus and extended by AMV reverse transcriptase (lane P). Lanes T, G, C, and A correspond to a dideoxy sequencing reaction performed with the same primer. The sequencing encompassing the initiation start (marked by an asterisk) is enlarged.

Mapping of \textit{znt} transcription start site. To define the transcription unit more precisely, the 5' end of \textit{zntR} transcript was mapped. A 19-base oligonucleotide specific to the coding region was annealed with total RNA and extended in a primer extension assay. The transcription start site was located in between the −10 region and an imperfect 8-2-8 hyphenated inverted repeat that represented a potential regulatory site (Fig. 4). The presence of a minor band in Fig. 4 (lane P) may be due to a minor transcription start site in the \textit{znt} operon, to the degraded mRNA product, or to RNA secondary structures.

Localization of \textit{znt} on the bacterial chromosome. Various known \textit{S. aureus} gene clones available in our laboratory were used to explore the location of \textit{znt} on the bacterial chromosome. These include \textit{lytT} (15), \textit{lytM} (24), \textit{all} (21), \textit{brnQ} (30), and others. Dot blot studies showed hybridization of \textit{znt} with the cosmid containing the \textit{lytT} gene, thus mapping it within the \textit{SmaI-B fragment of \textit{S. aureus}} (14).

In our initial studies, we found that many coagulase-positive and coagulase-negative strains of \textit{S. aureus} conferring resistance to zinc (∼4.0 mM) and cobalt (∼3.0 mM) ions. We have subsequently isolated a chromosomal fragment of \textit{S. aureus} con- ferring resistance to zinc and cobalt ions. Northern blot analysis indicated that the \textit{zntR} and \textit{zntA} genes were cotranscribed. Their transcription was inducible and dependent on the concentration of zinc ions. The mutational and complementation analyses of the \textit{zntA} gene demonstrated that the operon was involved in the transport of zinc and probably cobalt ions. The zinc transport analysis indicated that the \textit{zntA} mutant accumulated more zinc ion than did the wild-type strain. In addition, the resistance level of zinc and cobalt ions depended on the copy number of \textit{znt}. These results suggest that \textit{ZntA} is a structural gene which functions as a transporter of zinc and cobalt ions rather than the sensor of the two-component system. In mutational analysis, the insertion of a kanamycin resistance cassette was found to be close to the C-terminal end of \textit{ZntA}. Although the insertion fragment was read through, the resulting amino acid residues (data not shown) in the C terminus lacked the characteristic histidine-rich region, and the fusion protein thus was nonfunctional. These observations implied that the C terminus of \textit{ZntA}, which contains the histi- dine-rich region, is important for zinc transport.

Uptake and efflux of the heavy metal ions in bacteria are energy-dependent processes (27). However, no gene was found to be present in the staphylococcal \textit{znt} operon for the necessary energy-transducing systems for transporting zinc and cobalt ions. \textit{ZntA} also lacks the characteristic ATP-binding sites. No significant differences were observed in the growth kinetics of the mutant under normal physiological conditions, suggesting that \textit{zntA} may be dispensable in \textit{S. aureus}.

Our results indicate that we have cloned the gene responsible for the transport of zinc and probably cobalt ions in \textit{S. aureus} allowing tolerance to heavy metals. The \textit{zntA} gene codes for a transmembrane structural protein responsible for the efflux of zinc and cobalt ions. The \textit{zntR} gene also encodes a regulatory molecule which probably autoregulates its own expression. We are currently investigating the functional aspects of \textit{zntR}.

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