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Characterization of the Role of the Divalent Metal Ion-Dependent Transcriptional Repressor MntR in the Virulence of *Staphylococcus aureus*

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DtxR-type metal ion-dependent repressors, present in many bacterial pathogens, may regulate expression of virulence genes such as that encoding diphtheria toxin. SirR, a DtxR homologue initially identified in *Staphylococcus epidermidis*, governs the expression of the adjacent *sitABC* operon encoding a putative metal ion ABC transporter system. We identified a *sirR* homologue, *mntR*, in *Staphylococcus aureus* and demonstrated by gel shift assay that the corynebacterial repressor DtxR binds to the *S. aureus mntABC* operator in the presence of Fe^{2+} or Mn^{2+}. Since a mutant DtxR, DtxR(E175K), functions as an iron-independent hyperrepressor in certain settings, we constructed a heterodiploid *S. aureus* strain expressing *dtxR*(*E175K*) from the native *mntR* promoter. Transcription of the *S. aureus mntABC* operon was repressed in the presence of Fe^{2+} or Mn^{2+} in wild-type and heterodiploid *S. aureus* strains. Under metal ion-limiting conditions, *mntABC* transcription was reduced but not abolished in *S. aureus* isolates expressing *dtxR*(*E175K*) compared with an isogenic control, suggesting that DtxR(E175K) binds the *S. aureus* MntR box in vivo. Under all conditions tested, *mntABC* transcription in the *dtxR*(*E175K*)-expressing strain was reduced relative to the isogenic control, indicating that DtxR(E175K) function was constitutively active. In the mouse skin abscess model, *dtxR*(*E175K*)-expressing *S. aureus* recombinants showed significantly reduced CFU levels compared with the isogenic wild-type control. We conclude that the *S. aureus* MntR box is recognized by corynebacterial DtxR proteins and thus belongs to the DtxR family of metal-dependent operator sites. Moreover, constitutive repression by DtxR(E175K) reduces the virulence of *S. aureus* in the mouse skin abscess model.

Staphylococcus aureus is a major human pathogen that causes a variety of diseases ranging from minor skin ailments to life-threatening deep infections such as endocarditis, meningitis, arthritis, and toxic shock syndrome (16, 30, 40). Despite the introduction of new antibiotics, the morbidity and mortality from serious *S. aureus* infections remain high and drug resistance is emerging as a new threat (5). The identification and characterization of specific virulence genes continue to be a high priority, as these genes may prove to be important future drug targets.

The diphtheria toxin repressor, DtxR, from *Corynebacterium diphtheriae*, has been shown to be an iron-dependent repressor that controls the expression of a series of iron-sensitive genes, including the diphtheria toxin gene, the gene for heme oxygenase, and genes involved in siderophore production (1, 24, 27, 33). In addition to *C. diphtheriae*, DtxR homologues have been found in *Streptomyces* spp. (8), *Brevibacterium lactofermentum* (19), *Mycobacterium* spp. (6), *Streptococcus mutans* (13), *Treponema pallidum* (9), *Staphylococcus epidermidis* (11), and *S. aureus* (12). There is evidence to suggest that these DtxR homologues are important in regulating genes encoding metal ion transport systems related to virulence and oxidative stress defense. *S. epidermidis* SirR, *S. mutans* SloR, and *T. pallidum* TroR are known to bind a specific operator region that regulates an ATP-binding cassette (ABC) transporter system. The streptococcal DtxR homologue, SloR, has been found to regulate the *sloABC* operon, the latter being members of the lipoprotein receptor antigen I (LraI) family of ABC transporter systems. Interestingly, a mutation in the *S. mutans sloC* gene results in a loss of virulence in the rat model of endocarditis (13). Another LraI locus in *Streptococcus parasanguis*, known as *fimA*, has also been implicated in virulence in an endocarditis model (3).

Sun et al. isolated and characterized a series of *dtxR* mutants created by PCR mutagenesis and found that a single amino acid substitution of lysine for glutamic acid at position 175 [mutant DtxR(E175K)] conferred an iron-insensitive, hyperrepressor phenotype (31). Wild-type DtxR of *C. diphtheriae* requires Fe^{2+} ions to transcriptionally regulate the *tox* gene (33). Since DtxR in *C. diphtheriae* and its closest phylogenetic homologue, IdeR in *Mycobacterium tuberculosis* (28), are likely to be global regulators that control the expression of iron-responsive genes involved in iron acquisition and virulence, heterodiploids expressing DtxR(E175K) would be expected to have a dominant negative phenotype and to be constitutively unable to upregulate iron-repressed genes. Along these lines, Manabe

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et al. reported that an *M. tuberculosis* heterodiploid expressing DtxR(E175K) was attenuated compared with a wild-type strain in a mouse model of tuberculosis (17). In vitro, by gel shift assay, the DtxR protein was demonstrated to bind to putative IdeR-regulated genes. Likewise, IdeR binds to the *C. diphtheriae tox* promoter (28).

Horsburgh et al., using primers designed from the *S. epidermidis sirR* gene, isolated and sequenced the *S. aureus* homologue, which they termed *mntR* (12). In this study, using in vitro gel retardation assays, we demonstrate that DtxR (C102D), a biologically active iron-dependent repressor, binds to the *S. aureus* MntR box in the 5′ untranslated region of the *mntABC* operon of *S. aureus*. Furthermore, using reverse transcription (RT)-PCR and molecular beacons, we show that transcription of *mntABC* is repressed under conditions containing Fe^{2+} or Mn^{2+} ions. *S. aureus* expressing the metal ion-independent repressor DtxR(E175K) inhibited *mntABC* expression under conditions with and without iron. In addition, this strain was attenuated in a mouse skin abscess model compared with the parent strain containing an empty plasmid.

MATERIALS AND METHODS

Strains and plasmids. *S. aureus* RN4220 and RN6390 were gifts from Richard Novick. *S. aureus* was cultured at 37°C in tryptic soy broth or tryptic soy agar. *Escherichia coli* (DH5α) was cultured at 37°C in Luria-Bertani broth or on Luria-Bertani agar. Staphylococcal siderophore detection media (SSD) (2 mM KH_2PO_4, 7.9 mM NaCl, 17.2 mM NH_4Cl, 2% [vol/vol] 1.5 M Tris-HCl [pH 8.8] solution, 20 mM glucose, 0.6% [wt/vol] Casamino Acids [Difco], 39 μM tryptophan, 32 μM nicotinic acid, and 6 μM thiamine-HCl) were used as the minimal media for both iron-depleted and iron-rich media (15). The iron-depleted medium was prepared by treating the minimal medium with 10 g of Chelex 100 (Sigma) per liter and stirring for 1 h at room temperature to remove divalent and trivalent metal ions. The iron-rich medium was SSD medium containing ferric ammonium acetate at a final concentration of 50 μg/ml. Antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 5 μg/ml.

Construction of the *dtxR*(*E175K*) shuttle vector plasmid. The *mntR* gene of *S. aureus* was identified in The Institute of Genomic Research (TIGR, Rockville, Md.) database as a homologue of the *S. epidermidis sirR* gene (11). The first 10 codons of *dtxR*(*E175K*) from *C. diphtheriae* were altered to reflect *S. aureus* codon usage (Fig. 1A). The modified DNA fragment of *dtxR*(*E175K*) was amplified by PCR from pNBV1/SAD (17) with primers SADX F-1 (5′-CATATG AAAGACTTAGTGGACACAACAGAAATGTACTTGCGTACTATC) and SADXR-2 (5′-GTCGACTTAGAGTTCTTCGATACGAATAGT) (Fig. 1). The PCR products were cloned into pCRII (Invitrogen Corp., Carlsbad, Calif.), and the resulting plasmid was designated pSDTXR/PCRII. Two hundred base pairs of upstream sequence from *mntR* including the putative promoter was amplified with MntRPro F-1 (5′-GGATCCTTGCAGTTGTTGTTGTATAGG) and MntRPro R-2 (5′-CATATGACTTTCACCTCACATACATTG) and then cloned into pCRII, digested by *Bam*HI and *Nde*I, and ligated into the same sites in the pSDTXR/PCRII. The resulting plasmid was designated pSADX1. The plasmids were extracted by using the Qiagen (Chatsworth, Calif.) plasmid purification system. After a restriction digest by *Bam*HI and *Sal*I, the fragment encoding the putative *mntR* promoter and modified *dtxR*(*E175K*) were cloned into the pSPT181 multi-copy shuttle plasmid and designated pSADX. This construct was then transformed into the restriction-deficient RN4220 recipient by electroporation (22) and transduced at 37°C into RN6390 with phage 80α by the method described by Novick et al. (18). MA2004 is the heterodiploid *S. aureus* strain expressing *dtxR*(*E175K*) from the *mntR* promoter in recombinant pSADX, while MA2181 is the control *S. aureus* RN6390 containing only the parent shuttle vector pSPT181.

Cell extract preparation and Western blotting. The *S. aureus* MA2004 heterodiploid and MA2181 control strains were cultured in tryptic soy broth containing 5 μg of tetracycline/ml for 18 h. After pelleting, the cells were resuspended in 1.0 ml of TEG buffer (25 mM Tris-HCl, 5 mM EGTA [pH 8.0]), and cell extracts were prepared from lysostaphin-treated cells by using an adaptation of the method originally described by Schindler and Schuhardt (23). One microgram of whole-cell protein extract was separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Hybond; Amersham Pharmacia, Piscataway, N.J.) by the semidry technique (Transblot SD; Bio-Rad, Hercules, Calif.). The blot was blocked with 5% nonfat milk in phosphate-buffered saline with 0.1% Tween 20 for 1 h and incubated at 4°C with anti-DtxR polyclonal antibodies (35). After being washed, the membrane was incubated with horseradish peroxidase-conjugated antirabbit antibody diluted in phosphate-buffered saline with 0.1% Tween 20 at room temperature for 2 h. After being washed, the Supersignal chemiluminescent substrate (Pierce, Rockford, Ill.) was used for autoradiograph development.

DNA gel shift assay. The gel electrophoresis mobility shift assay (EMSA) with purified DtxR protein was performed as previously described (33). Radiolabeled *mntABC* promoter/operator region DNA containing the MntR box was generated by PCR by using 100 ng of ^{32}P-end-labeled primer mixed with 150 ng of unlabeled primer and template DNA from gel-purified 100-bp cold fragments containing the MntR box PCR, amplified by using primers 5′-CTCTTTTTCTT CAGTTAACATACT and 5′-GCCGCGTACTGGTATCGATAAGGA. Binding reactions were carried out with 10 mM Tris-HCl (pH 7.4)–5 mM $MgCl_2$–50 mM KCl–1 mM dithiothreitol–5% glycerol–50 μg of calf thymus DNA/ml–5 μg of bovine serum albumin. Freshly prepared $FeSO_4$ or $MnSO_4$ was added at 125 μM. For the divalent metal ion-free sample, the divalent metal iron chelator EDTA at a concentration of 0.1 mM was added to the reaction solution. Binding reactions were equilibrated for 30 min. Samples were immediately submitted to electrophoresis at 200 V on a 5% nondenaturing polyacrylamide gel in 0.5× Tris-borate EDTA buffer. For the MntR EMSA, purified MntR recognized a 106-bp α-^{32}P-labeled PCR product containing the 5′promoter/operator (P/O) region of the *S. aureus mnt*ABC P/O. The end-labeled fragments (20 fmol) were incubated for 15 min at room temperature with either purified MntR (100 ng) or His-tagged MntR (100 ng). To determine the metal-dependent nature of MntR binding to the *mnt*ABC, P/O samples were incubated prior to resolution by native gel electrophoresis in the presence of EDTA (0.5 mM).

RT and real-time PCR. RNA was extracted from *S. aureus* grown for 24 to 36 h under iron-rich or iron-deleted conditions in minimal medium containing 50 μM $MgCl_2$ with an RNeasy RNA kit (Qiagen, Inc.). Before cell lysis, the *S. aureus* was incubated in Tris-EDTA buffer with 50 μg of lysostaphin (Sigma, St. Louis, Mo.)/ml for 10 min at 37°C followed by silica bead beating to destroy the cell walls. The contaminating DNA in 10 μl of total RNA was digested by 2 μl of DNase I (Ambion). First-strand cDNA was synthesized from 1 μg of total RNA by incubation with *mntABC* reverse primer at 65°C for 15 min, followed by standing on ice for 5 min. RNA was incubated in reverse transcriptase buffer, 5 mM concentrations of deoxynucleoside triphosphates, and 16 U of murine leukemia virus reverse transcriptase (Roche) at 37°C for 1 h. Aliquots of first-strand cDNA were used in the PCR.

Molecular beacons were designed based on the specific 190-bp sequence of *mntABC* of *S. aureus* by using an in silico algorithm. A DNA folding program to estimate the stability of the hairpin stem (now available at http://www.cbr.nrc.ca/services/dnafold_e.php) was used. Each 50-μl reaction mixture contained a 0.2 μM concentration of the primer set, 100 μM concentrations of deoxynucleoside triphosphates, 1 U of Platinum *Taq* DNA polymerase (Life Technology), 3 mM $MgCl_2$, 500 mM KCl, 10 mM Tris-HCl (pH 8.4), and 2.5 μl of reverse-transcribed sample. The PCRs were performed on the iCycler (Bio-Rad) with the following cycle conditions: preincubation at 65°C for 10 min followed by denaturation at 94°C for 2 min, and then 32 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 1 min, and extension at 72°C for 30 s, followed by a final extension at 72°C for 10 min. Fluorescence was monitored during the annealing step.

Mouse skin abscess model. Hairless but immunocompetent SKH1 male mice, 6 weeks of age, were anesthetized and injected subcutaneously with the MA2181 control and the MA2004 heterodiploid strains of *S. aureus*. There were five mice in each group. After 7 days bacterial numbers in each lesion were measured by CFU counts by using a previously described method (2, 4). Significant differences between groups were assessed by nonparametric statistics. Animals were treated according to the American Association for Accreditation of Laboratory Animal Care guidelines under a study protocol approved by the appropriate institutional review board.

RESULTS

Identification of the *S. aureus mntR* gene. Using the 19-bp palindromic consensus sequence for DtxR identified in *C. diphtheriae* (5′-TTAGGTTAGCCTAACCTAA-3′), we found a similar sequence that corresponded with 16 bp of the 19-bp DtxR-box consensus sequence in contig 6186 from the then-unfinished *S. aureus* genome sequence data provided by TIGR

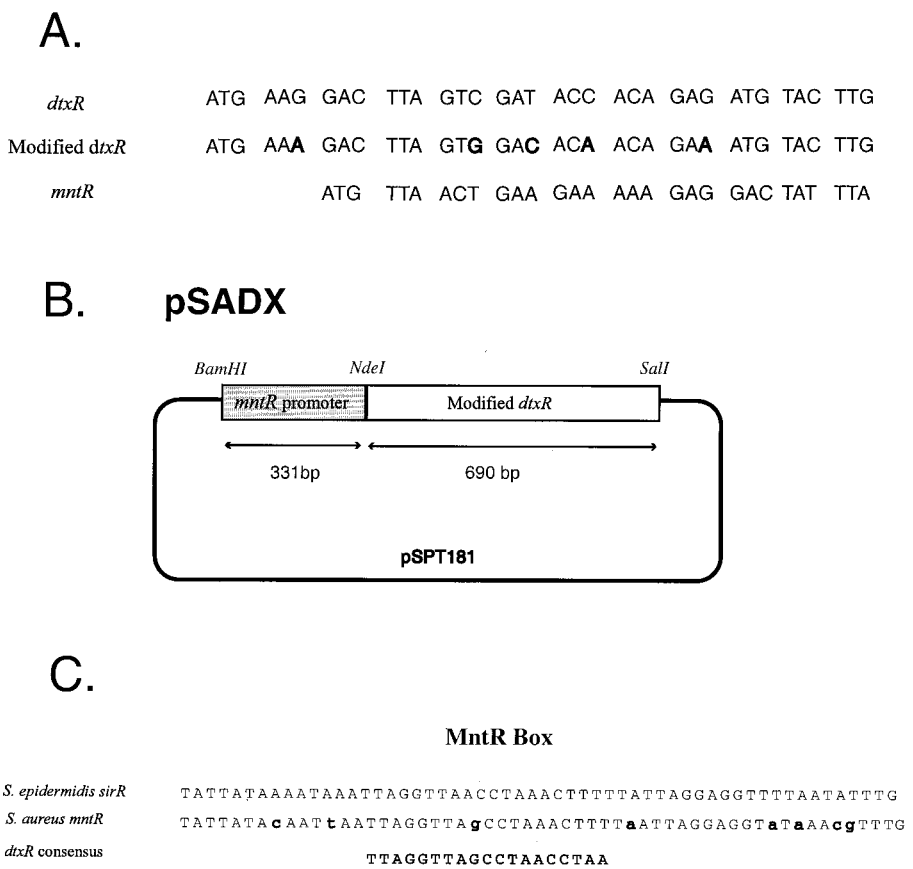

FIG. 1. (A) Sequences of the initial 10 or 12 codons of *dtxR*, modified *dtxR* (modified to *S. aureus* codon preferences), and *mntR*. (B) Schematic representation of the pSADX shuttle vector plasmid containing the *mntR* promoter and the modified *dtxR*. (C) The MntR box. The alignment of the *S. aureus* MntR box with the *S. epidermidis* SirR box (11) and the consensus DtxR box (14) is shown.

(http://www.tigr.org) (14). Interestingly, this sequence is identical to the SirR box upstream of the *S. epidermidis sitABC* gene (11) (Fig. 1C). Moreover, a BLAST search of the downstream region revealed three open reading frames with strong matches to the *sitABC* operon of *S. epidermidis*. The three encoded proteins, now designated MntA, MntB, and MntC, have 83, 95, and 83% similarity and 67, 83, and 72% identity, respectively, with *S. epidermidis* SitA, SitB, and SitC. The *S. aureus* operon has the same orientation and organization as previously reported for the *S. epidermidis sitABC* operon (13).

DtxR(C102D) binds to the MntR box in the promoter region of *mntABC* in *S. aureus*. To examine whether the DtxR-related proteins are capable of binding to the putative MntR box, a DNA gel shift binding assay was done. DtxR(C102D), in which a cysteine is changed to an aspartate at position 102, was used because of greater stability and ease of purification. Both DtxR and DtxR(C102D) function as iron-dependent repressors. Although the regulatory activity of DtxR(C102D) is not as sensitive to the concentration of iron as is that of DtxR, it is able to suppress the expression of the *tox* gene (34). A 200-bp ^{32}P-labeled MntR box upstream of the *mntABC* operon was amplified by PCR and incubated with DtxR(C102D) in the presence or absence of Fe^{2+} or Mn^{2+}. The MntR box shifted in a metal ion-dependent manner with both Mn^{2+} and Fe^{2+} (Fig. 2A). Furthermore, the addition of EDTA, a divalent metal ion chelator, to the running buffer abolished binding even in the presence of metal ions (Fig. 2B and data not shown).

Expression of the constitutively active DtxR(E175K) hyperrepressor in *S. aureus*. In order to express the *C. diphtheriae dtxR*(*E175K*) gene efficiently in *S. aureus*, we altered the codon usage of the first 10 codons of the *dtxR*(*E175K*) gene to *S. aureus* codon preferences and fused the modified gene to a 331-bp DNA fragment containing the native *S. aureus mntR* promoter and 5′ untranslated regulatory sequences. The resulting shuttle vector, pSADX, derived from pSPT181 (Fig. 1A and B), was transformed into the parent *S. aureus* strain RN6390 to produce the MA2004 heterodiploid strain. We confirmed that this strain expressed the 25.3-kDa protein by using anti-DtxR polyclonal antibodies in Western blotting (Fig. 3, lane 2). Purified DtxR run on the same gel was also detected by using this antibody (Fig. 3, lane 1), whereas the parent strain containing only an empty plasmid, i.e., the MA2181 control, showed no immunoreactivity by Western blotting (Fig. 3, lane 3).

Transcription of *mntABC* is divalent metal ion dependent by RT-PCR. Previous studies have shown that the expression of *S. aureus mntABC* is MntR dependent and similarly that the expression of *S. epidermidis sitABC* is SirR dependent (11, 12). Hence, to evaluate the repressor function of DtxR(E175K) in

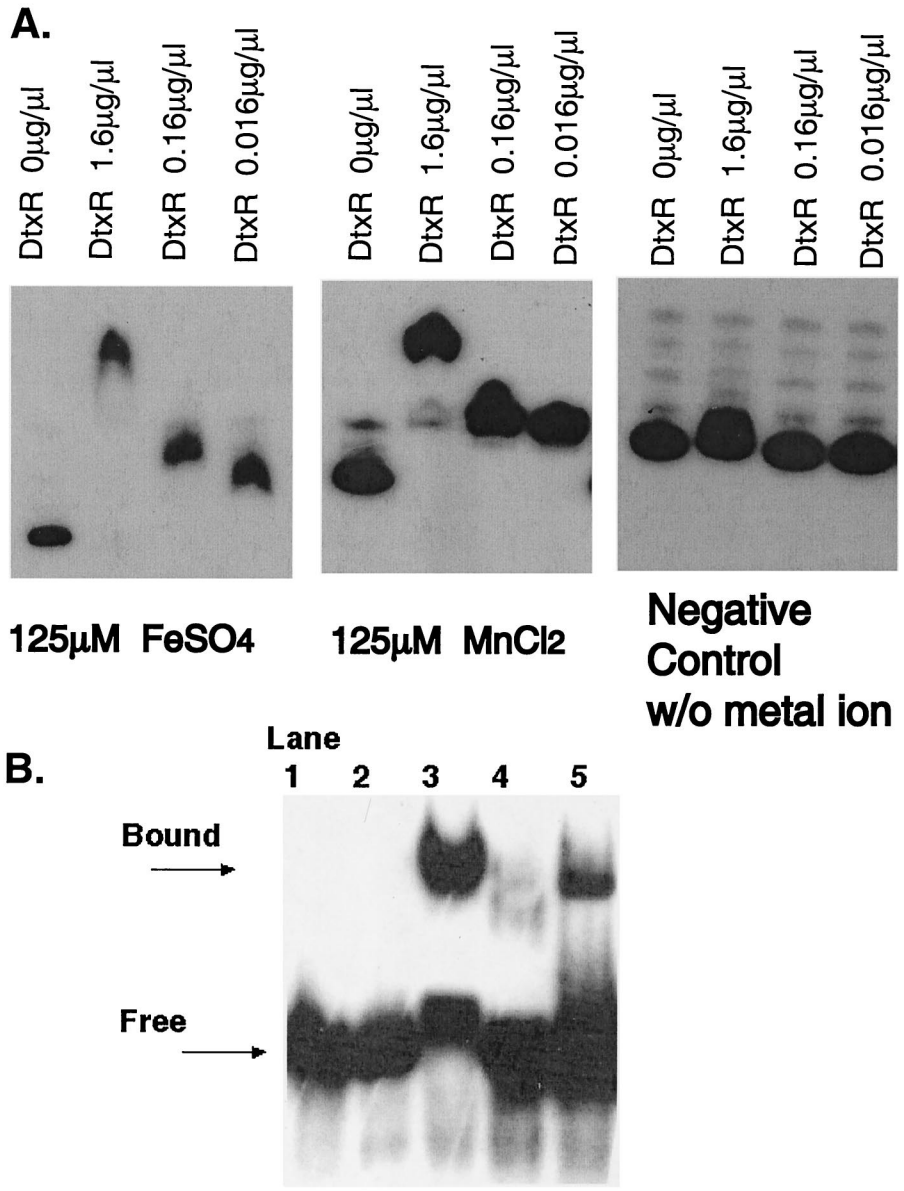

FIG. 2. DtxR and MntR recognize the putative MntR box, and binding is metal ion dependent. (A) EMSA showed that increasing concentrations of DtxR in the presence of a 125 μM concentration of either $FeSO_4$ (left panel) or $MnCl_2$ (middle panel) bound to and shifted the MntR box in a dose-dependent manner. In the absence of metal ion (right panel), no shift was seen when DtxR was added to the MntR box. (B) EMSA with MntR demonstrated that divalent cations are required for binding and shifting of the MntR box. Lane 1, *S. aureus mntABC* promoter/operator (P/O); lane 2, *mntABC* P/O (in excess) with His-tagged MntR and 0.5 mM EDTA; lane 3, *mntABC* P/O with His-tagged MntR; lane 4, *mntABC* P/O with untagged MntR and 0.5 mM EDTA; lane 5, *mntABC* P/O with untagged MntR.

the *S. aureus* heterodiploid strain MA2004, we chose to monitor *mntABC* transcriptional levels. RT-PCR using molecular beacons was carried out to quantify transcription of the *mntABC* operon. A 190-bp segment in *mntB* was selected by a computer algorithm as the best molecular beacon target sequence for *mntABC*. RNA samples from the *S. aureus* MA2181 control (RN6390 transformed with pSPT181) and from the MA2004 heterodiploid (RN6390 transformed with pSADX) were grown for 24 h in 125 μM $FeSO_4$, 125 μM $MnCl_2$, or Fe^{2+}- and Mn^{2+}-restricted medium. As may be seen in Table 1, the empty vector control strain MA2181 revealed a 78-fold derepression of *mntABC* transcription upon shifting from Mn^{2+}-replete conditions to divalent metal ion-free conditions (0.5 ± 0.15 to 38.8 ± 4.14 pmol [means ± standard deviations]). Similarly, iron withdrawal led to a 491-fold derepression of *mntABC* transcription (0.079 ± 0.017 to 38.8 ± 4.14 pmol). Thus, in the control strains *mntABC* transcription appears to be dependent on either Mn^{2+} or Fe^{2+}, as was observed by Horsburgh et al. (12). Addition of the constitutively active *dtxR*(*E175K*) gene in the heterodiploid strain MA2004 produced a reduction in the amount of derepression by iron or manganese withdrawal but did not fully achieve constitutively active repression. Withdrawal of Mn^{2+} produced a 24-fold derepression in contrast to the 78-fold derepression with the

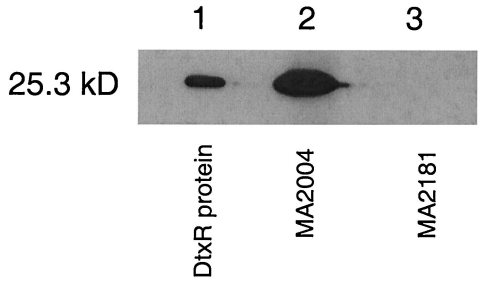

FIG. 3. Expression of the 25.3-kDa DtxR protein by the *S. aureus* MA2004 heterodiploid. The DtxR protein produced from the *mntR* promoter in the heterodiploid (lane 2) and native (lane 1) DtxR is recognized by an anti-DtxR antibody by Western blot analysis. The parent *S. aureus* strain containing an empty plasmid does not make a detectable cross-reacting protein, such as MntR, PerR, Zur, or Fur, the endogenous Fe-dependent regulators in *S. aureus* (lane 3).

control strains (0.12 ± 0.008 to 2.91 ± 0.9 pmol). Likewise, withdrawal of iron led to a significant increase, from undetectable levels of transcription to 2.91 pmol. Comparing the amount of additional repression achieved by expression of the constitutively active *dtxR*(*E175K*) gene in the heterodiploid strain MA2004 in the presence of Mn^{2+} or Fe^{2+}, there was a 4.2- or >10.4-fold increase in repression mediated by the presence of the constitutively active repressor, respectively. Under Fe^{2+}-and Mn^{2+}-free conditions, the repression mediated by the presence of DtxR(E175K) increased 13.3-fold (Table 1). Table 1 shows that under iron-replete conditions, the wild-type control strain MA2181 showed some degree of *mntABC* transcription (0.079 ± 0.017 pmol) while the heterodiploid MA2004 containing the constitutively active *dtxR*(*E175K*) gene repressed transcription of *mntABC* to <0.0076 pmol, which was below the limit of detection of our highly sensitive RT-PCR molecular beacon assay.

Virulence assessment of the DtxR(E175K)-containing *S. aureus* strain by use of the mouse skin abscess model. To evaluate whether MntR-mediated divalent cation-independent gene regulation plays a role in the virulence of *S. aureus*, we evaluated the pathogenicity of our empty vector control strain MA2181 and the heterodiploid MA2004 strain containing the constitutively active *dtxR*(*E175K*) gene. We postulated that the heterodiploid strain MA2004 might show a virulence defect, because our earlier data indicated that the presence of the *dtxR*(*E175K*) gene altered the ability of *S. aureus* to derepress *mntR*-dependent gene expression following a shift into Fe^{2+}- and Mn^{2+}-free media. A comparison of virulence between the MA2004 heterodiploid and the MA2181 control strain was accomplished by using the murine skin abscess model of infection. The inocula of MA2004 and MA2181 were 1.1×10^8 CFU and 0.6×10^8 CFU, respectively. Seven days after infection, a statistically significant difference between the mean CFU of the MA2181 control strain and the mean CFU of the MA2004 heterodiploid was recovered from each lesion ($1.04 \times 10^8 \pm 0.46 \times 10^8$ CFU of MA2181 and $1.41 \times 10^6 \pm 0.96 \times 10^6$ CFU of MA2004) (Fig. 4).

TABLE 1. Quantitative *mntABC* transcriptional levels in *S. aureus* strains exposed to different divalent metal ion concentrations

Metal ion (concn)	Mean ± SD (pmol) of *mntABC* transcriptional levels in:		Ratio
	MA2181 empty vector control (*S. aureus* pSPT181)	MA2004 heterodiploid [*S. aureus* pSPT181-P_{mntR}::*dtxR*(*E175K*)]	
Fe^{2+}- and Mn^{2+}-free	38.8 ± 4.14	2.91 ± 0.9	13.3:1
Fe^{2+} (125 μM)	0.079 ± 0.017	<0.0076[a]	>10.4:1
Mn^{2+} (125 μM)	0.5 ± 0.15	0.12 ± 0.008	4.2:1

[a] Undetectable; the lower limit of detection was 0.0076 pmol.

DISCUSSION

In 1936 Pappenheimer and Johnson demonstrated that the addition of low concentrations of iron to the growth medium resulted in inhibition of toxin production by *C. diphtheriae* (20). Uchida et al. demonstrated that the diphtheria toxin structural gene, *tox*, was carried by corynebacteriophage β and that the addition of iron results in the immediate repression of toxin synthesis (38). Boyd et al. and Schmitt and Holmes described the molecular cloning of an iron-dependent regulatory element, *dtxR* (diphtheria toxin repressor), from genomic libraries of nontoxigenic *C. diphtheriae* (1, 27). DtxR can specifically bind to the palindromic sequence in the *tox* operator in the presence of iron and thereby repress the transcription of the *tox* gene. At low iron concentrations, DtxR is believed to undergo a conformational change which reduces its affinity for the *tox* operator and leads to derepression of *tox* transcription (37).

Recently, a single missense mutation was identified by PCR mutagenesis using a positive genetic selection system to clone *dtxR* alleles with hyperrepressor phenotypes. One of the mutant proteins encoded by this family of mutant alleles, DtxR(E175K), was found to bind to the *tox* operator site and function as an iron-independent repressor (31). At least seven promoters are already known to be negatively regulated by DtxR (14, 24–26, 29, 32) in *C. diphtheriae*. These promoters are the iron-regulated promoters (IRPs), designated IRP1 through IRP5, as well as the promoters for *tox* and heme oxygenase (*hmuO*). DtxR acts as a global regulatory protein to control the expression of diphtheria toxin, to regulate the high-affinity iron uptake system, and to govern expression of other iron sensitive genes.

Iron, in vivo, normally remains bound to host proteins such as ferritin, transferrin, lactoferrin, hemoglobin, and iron sulfur proteins; only a minor fraction (10 to 18%) is available in the free form (7, 36). Because free iron is scarce in mammalian hosts, it has been postulated that a shift into an iron-restrictive environment prompts not only the transcription of genes for high-affinity iron acquisition but also the transcription of virulence genes which enhance microbial survival within the host in pathogenic bacteria. The construction of heterodiploid bacteria in which one DtxR allele is a hyperrepressor would be anticipated to interfere with the normal induction of such genes at low iron concentrations, resulting in an attenuation of virulence in pathogenic bacteria such as *S. aureus*. This concept was supported by the work of Manabe et al., in which an *M. tuberculosis* DtxR(E175K)-expressing heterodiploid strain was attenuated compared to the wild-type control in a mouse model. These results suggested that the iron-dependent re-

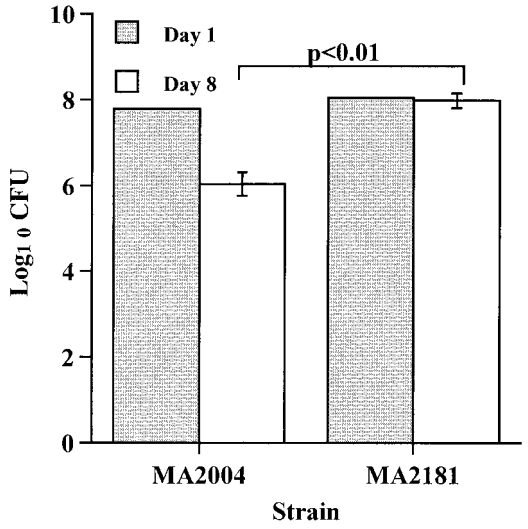

FIG. 4. The *S. aureus* MA2004 heterodiploid expressing DtxR is significantly less virulent in a mouse skin abscess model than is a strain carrying the empty MA2181 plasmid, suggesting that the MutR-mediated gene regulation is essential for virulence in this model.

pressor IdeR may control genes essential for virulence in *M. tuberculosis* (17).

S. epidermidis has a *sitABC* operon, which works as an ATP binding cassette transporter, and a staphylococcal iron regulator repressor, SirR, with homology to DtxR, the gene for which is immediately upstream of the *sitABC* operon and is divergently transcribed as a 645-bp open reading frame. Since SirR can bind to the *sitABC* operator site (the SirR box) in the presence of ferrous or manganese ions, it appears to be an iron-dependent repressor of *sitABC* (11). *S. aureus* has recently been shown to have a homologous locus known as *mntR* and *mntABC* (12). The MntA, MntB, and MntC proteins share 83, 95, and 83% similarity and 67, 83, and 72% identity with *S. epidermidis* SitA, SitB, and SitC, respectively.

S. aureus MntA has a consensus ATP binding motif which includes a P loop (GPNGAGKA) (21). MntC has a consensus prelipoprotein signal peptide cleavage sequence of staphylococcal lipoprotein (ILAACG) at position 14 (10). Upstream of *mntABC*, there is strong homology with the repressor binding site, an 18- to 19-bp putative MntR box sequence in *S. aureus* closely related to the SirR box consensus in *S. epidermidis* and the DtxR consensus sequence in *C. diphtheriae*. Using allelic replacement to inactivate *mntR*, *mntA*, and *mntH*, Horsburgh et al. showed that the mutants have defective growth in the absence of metal and an increased susceptibility to intracellularly generated superoxide radicals due to a diminished uptake of manganese. They clearly demonstrated that MntR acts as a negative and positive regulator of *mntABC* and *mntH*, respectively (12).

In this study DtxR(C102D) was substituted for wild-type DtxR and used for EMSA in order to assess whether the DtxR-like proteins can bind to the *S. aureus* MntR box. Analysis of point mutations at residue 102 showed that only Asp can substitute for Cys to generate an active DtxR protein (34). However, the iron-dependent regulatory activity of DtxR (C102D) is not as strong as that of wild-type DtxR. Our data demonstrate that the DtxR(C102D) does indeed bind to the *S. aureus* MntR box in the presence of Fe^{2+} or Mn^{2+} ions, and the affinity seems to be related to the concentration of these metal ions. These data indicate that DtxR-related proteins, as well as MntR, may act as a repressor and control the expression of *mntABC*.

Real-time RT-PCR was carried out to identify the transcriptional levels of an *mntABC* mRNA under iron-free, high-ferric ion, or high-manganese ion conditions. To quantify the reverse transcripts, we employed real-time PCR using molecular beacons (39). Under manganese- and iron-free conditions, the MA2181 control strain expressed 38.8 ± 4.14 pmol of *mntABC* whereas the MA2004 heterodiploid expressed 2.91 ± 0.9 pmol of *mntABC*. These results confirmed the activity of DtxR(E175K) as an iron-insensitive hyperrepressor in *S. aureus*. In the presence of 125 μM ferrous sulfate, the level of expression of *mntABC* in the control strain MA2181 was 0.079 ± 0.017 pmol and the level in the MA2004 heterodiploid was below the level of detection. In the presence of 125 μM manganese ion, transcription in the MA2181 control strain was 0.5 ± 0.15 pmol and in the MA2004 heterodiploid strains it was 0.12 ± 0.008 pmol. These data show that *mntABC* transcription was repressed in both the wild-type and heterodiploid backgrounds and that the repression was stronger in the heterodiploid. DtxR(E175K) is deduced to be a repressor of the *tox* gene in iron-free conditions but in this study failed to fully repress the *mntABC* gene. This partial activity of DtxR(E175K) in *S. aureus* suggests that differences between the DtxR consensus binding sequence of *C. diphtheriae* and that of the *S. aureus* MntR box may influence the affinity of the foreign DtxR protein for DNA binding in *S. aureus*. These data further demonstrate that *S. aureus mntABC* transcription is Mn^{2+} dependent, suggesting that *mntR* is responsive to either Mn^{2+} or Fe^{2+} levels, as fully described by Horsburgh et al. (12).

For the virulence study, we used the subcutaneous abscess assay on hairless mice. On day 1, *S. aureus* was inoculated subcutaneously; the lesions were extracted on day 8, and colonies were counted. Bacterial survival by CFU counts of the MA2004 heterodiploid was statistically significantly less than that of the MA2181 control strain. In terms of the abscess size, MA2004 tended to cause smaller lesions than MA2181. However, the appearance of the lesions caused by the different strains was almost the same. Future experiments using different doses of heterodiploids could more clearly resolve these differences. This result indicates that DtxR(E175K) can attenuate the virulence of *S. aureus*. Also, *mntABC* or other genes regulated by MntR or DtxR may be related to virulence, since the expression of DtxR(E175K) reduced the virulence of *S. aureus*. Alternatively, the mutation in the repressors may affect growth or survival in the metal-limited environment found in mammalian hosts.

An earlier study in which *S. aureus mntR* was disrupted showed that there was no attenuation in the skin abscess model (12). The *mntR* mutant strain showed high-level expression of MntR-dependent genes irrespective of metal ion concentration. In contrast, our study, in which a constitutively active repressor DtxR(E175K) was added, revealed that *mntABC* transcription was significantly reduced at all metal ion concentrations, and especially under Fe^{2+}-replete conditions. Thus, an inability to derepress MntR-dependent genes during infec-

tion (when free metal ion concentrations are believed to be low) attenuates *S. aureus* virulence, while constitutive derepression, as in the case of *mntR* mutation, does not. The findings also suggest that activators which interfere with metal ion release and derepression in repressors such as MntR may have novel antibacterial effects.

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