Staphylococcus aureus NrdH Redoxin Is a Reductant of the Class Ib Ribonucleotide Reductase

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Staphylococci contain a class Ib NrdEF ribonucleotide reductase (RNR) that is responsible, under aerobic conditions, for the synthesis of deoxyribonucleotide precursors for DNA synthesis and repair. The genes encoding that RNR are contained in an operon consisting of three genes, nrdIEF, whereas many other class Ib RNR operons contain a fourth gene, nrdH, that determines a thiol redoxin protein, NrdH. We identified a 77-amino-acid open reading frame in Staphylococcus aureus that resembles NrdH proteins. However, S. aureus NrdH differs significantly from the canonical NrdH both in its redox-active site, C-P-P-C instead of C-M/V-Q-C, and in the absence of the C-terminal [WF]SGFRP[DE] structural motif. We show that S. aureus NrdH is a thiol redox protein. It is not essential for aerobic or anaerobic growth and appears to have a marginal role in protection against oxidative stress. In vitro, S. aureus NrdH was found to be an efficient reductant of disulfide bonds in low-molecular-weight substrates and proteins using dithiothreitol as the source of reducing power and as an effective reductant for the homologous class Ib RNR employing thioredoxin reductase and NADPH as the source of the reducing power. Its ability to reduce NrdEF is comparable to that of thioredoxin-thioredoxin reductase. Hence, S. aureus contains two alternative thiol redox proteins, NrdH and thioredoxin, with both proteins being able to function in vitro with thioredoxin reductase as the immediate hydrogen donors for the class Ib RNR. It remains to be clarified under which in vivo physiological conditions the two systems are used.

Ribonucleotide reductases (RNRs) are essential enzymes in all living cells, providing the only known de novo pathway for the biosynthesis of deoxyribonucleotides. These enzymes catalyze the controlled reduction of all four ribonucleotides to diphosphate, thus maintaining a balanced pool of deoxyribonucleotides during the cell cycle. Three main classes of RNRs are known. Class I RNRs are oxygen-dependent enzymes, class II RNRs are oxygen-independent enzymes, and class III RNRs are oxygen-sensitive enzymes. Class I RNRs are divided into two subclasses, subclasses Ia and Ib.

Staphylococcus aureus is a Gram-positive facultative aerobe and a major human pathogen (24). S. aureus contains class Ib and class III RNRs, which are essential for aerobic and anaerobic growth, respectively (26). The class Ib NrdEF RNR is encoded by the nrdE and nrdF genes: NrdE contains the substrate binding and allosteric binding sites, and NrdF contains the catalytic site for ribonucleotide reduction. The S. aureus NrdE and NrdF genes form an operon containing a third gene, nrdI, the product of which, NrdI, is a flavodoxin (5, 33). Many other bacteria such as Escherichia coli (16), Lactobacillus lactis (17), and Mycobacterium and Corynebacterium spp. possess class Ib RNR operons that contain a fourth gene, nrdH (30, 44, 50), whose product, NrdH, is a thiol-disulfide redoxin (16, 17, 40, 43, 49). More-complex situations are found for some bacteria where the class Ib RNR operon may be duplicated and one or more of the nrdI and nrdH genes may be missing or located in another part of the chromosome (29).

NrdH proteins are glutaredoxin-like protein disulfide oxidoreductases that alter the redox state of target proteins via the reversible oxidation of their active-site dithiol proteins. NrdH proteins function with high specificity as electron donors for class I RNRs (9, 16–18). They are widespread in bacteria, particularly in those bacteria that lack glutathione (GSH), where they function as a hydrogen donor for the class Ib RNR (12, 16, 17). In E. coli, which possesses class Ia and class Ib RNRs, NrdH functions in vivo as the primary electron donor for the class Ib RNR, whereas thioredoxin or glutaredoxin is used by the class Ia NrdAB RNR (12, 17). NrdH redoxins contain a conserved CXXC motif, have a low redox potential, and can reduce insulin disulfides. NrdH proteins possess an amino acid sequence similar to that of glutaredoxins but behave functionally more like thioredoxins. NrdH proteins are reduced by thioredoxin reductase but not by GSH and lack those residues in glutaredoxin that bind GSH and the GSH binding cleft (39, 40). The structures of the E. coli and Corynebacterium ammoniagenes NrdH redoxins reveal the presence of a wide hydrophobic pocket at the surface, like that in thioredoxin, that is presumed to be involved in binding to thioredoxin reductase (39, 40). NrdI proteins are flavodoxin proteins that function as electron donors for class Ib RNRs and are involved in the maintenance of the NrdF dithiorectol radical (5, 33). In Streptococcus pyogenes, NrdII is essential for the activity of the NrdHEF system in a heterologous E. coli in vivo system.
with the addition of ampicillin (100 μg ml⁻¹) or kanamycin (50 μg ml⁻¹) as needed. The effect of the oxidative stress compounds diamide, menadione, hydrogen peroxide, and tert-butyl hydroperoxide on the growth of *S. aureus* strains was determined as previously described (46).

**DNA manipulations.** For *E. coli*, the preparation of plasmids, DNA manipulations, and transformation of competent cells were performed as described previously (35). For *S. aureus*, Southern analysis and the transformation of competent cells were performed as described previously (26). Standard procedures were employed for restriction enzyme digestion, ligation, and PCR (35). Oligodeoxynucleotide primers used in this study are listed in Table S3 in the supplemental material.

**Construction of the nrdH deletion mutant.** A gene replacement strategy was used to create an *nrdH* knockout in *S. aureus* RN4220. The ~1.2-kb DNA region upstream of *nrdH* was amplified by PCR using primers (Up_For and Dw_For [see Table S3 in the supplemental material]) containing Pacl and BamHI restriction sites and [see Table S3 in the supplemental material]) containing Pacl and BamHI restriction sites and the DNA downstream of the *nrdH* region; *Ap* Em', Ap', Km', Km' (pKm1) BamHI-Bsp120I-digested pUP and *rB* positive); Apr Emr Km r This study

**TABLE 1. Bacteria, phage, and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strains, phage, or plasmid</th>
<th>Relevant characteristic(s)a</th>
<th>Source and/or reference(s)</th>
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<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
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</tr>
<tr>
<td>XL1-Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hisD17 supE44 relA1 lac [F' proAB lacI4 lacZΔM15 Tn10(Tc')]</td>
<td>Laboratory stock</td>
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<tr>
<td>HB101</td>
<td>supE44 hsdS20(rB− mB−) recA ana-14 proA2 lacY1 galK2 repL20 xyl-5 mtl-1</td>
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</tr>
<tr>
<td>BL21(DF3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Wild-type strain UV cured of prophages; spontaneous mutant with an 11-bp deletion in the <em>rsbU</em> gene (αB negative)</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>8325-4</td>
<td>Derivative of 8325-4 carrying the intact <em>rsbU</em> gene (αB positive); <em>rsbU</em>+</td>
<td>Gift of S. Foster; 15</td>
</tr>
<tr>
<td>SH1000</td>
<td>Restriction mutant of 8325-4 used as a primary recipient for plasmids propagated in <em>E. coli</em></td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>RN4220</td>
<td>*RN4220 in which the <em>arcR</em> gene was deleted and replaced by the Δkm-2 kanamycin cassette; Km'</td>
<td>This study</td>
</tr>
<tr>
<td>SH1000 ΔnrdH</td>
<td>ΔnrdH × SH1000ΔnrdH; Km'</td>
<td>This study</td>
</tr>
<tr>
<td>Phage φ11</td>
<td>Transducing phage that can infect <em>S. aureus</em> 8325-4</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>EcoRV-linearized pGEM vector with added 3'-terminal T bases; <em>Ap</em></td>
<td>Promega</td>
</tr>
<tr>
<td>pBR322::flkm-2</td>
<td>pBR322 carrying the omega-Km2 cassette; <em>Ap</em> Km'</td>
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<tr>
<td>pMUTIN-4</td>
<td>pUC18-based suicidal vector for Gram-positive bacteria; Pqna-ac lacZ Ppov-lac</td>
<td>47</td>
</tr>
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<td>pUP</td>
<td>Pacl-BamHI-digested pMUTIN-4 containing ~1.2 kb PCR amplified upstream of the <em>nrdH</em> region; <em>Ap</em> Em’</td>
<td>This study</td>
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<tr>
<td>pUP-DW</td>
<td>BamHI-Bsp120I-digested pUP and ~1.2-kb PCR-amplified 3’ end downstream of the <em>nrdH</em> region; <em>Ap</em> Em’</td>
<td>This study</td>
</tr>
<tr>
<td>pUP_Km DW (pKm1)</td>
<td>BamHI-digested pUP-DW containing the BamHI-flanked omega-Km2 cassette; <em>Ap</em> Em’ Km’</td>
<td>This study</td>
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<td>Novagen</td>
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<td>This study</td>
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<tr>
<td>pET-nrdE</td>
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<tr>
<td>pET-nrdF</td>
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<td>This study</td>
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<tr>
<td>pOUA</td>
<td>pET-14b(+)::<em>nrdE</em> (N-terminal His tag); <em>Ap</em></td>
<td>O. Uziel et al., unpublished data</td>
</tr>
<tr>
<td>pOUB</td>
<td>pET-14b(+)::<em>nrdB</em> (N-terminal His tag); <em>Ap</em></td>
<td>Uziel et al., unpublished data</td>
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a *Ap*, Em’, Km’, and Tc’, resistance to ampicillin, erythromycin-lincomycin, kanamycin, and tetracycline, respectively.
b Transduction with phage φ11 from donor × recipient.

**MATERIALS AND METHODS**

**Strains, media, and culture conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were grown at 37°C under aerobic and anaerobic conditions in tryptone soy broth (TSB; Difco) as described previously (26). When appropriate, kanamycin (200 μg ml⁻¹) or erythromycin (12 μg ml⁻¹) was supplemented. Phage transductions were carried out with φ11 as described previously (26). *E. coli* cells were grown in Luria-Bertani (LB) medium
mycin and kanamycin. The expected single integration event resulting from recombination between the pUP_Km_DW construct (pKm1) and the host chromosomal region was confirmed by PCR and Southern analysis (data not shown). To select for spontaneous segregation of the wild-type nrdH allele, one transformant was propagated for ~100 generations in TSB liquid medium containing kanamycin but lacking erythromycin, and colonies were screened for a loss of the erythromycin marker on TSB plates containing kanamycin. One kanamycin-resistant, erythromycin-sensitive clone was chosen for further analysis by PCR (primers For_del_of_nrdH and Rev_del_of_nrdH [Table S3]) and DNA sequencing to confirm the desired deletion-substitution into the nrdH gene. Transduction with phage λ11 was used to introduce the deletion-substitution mutation into S. aureus SH1000 ( rekB) as described previously (26).

Cloning and expression. The S. aureus nrdH, nrdE, and nrdF genes were amplified from genomic DNA by PCR using the primers listed in Table S3 in the supplemental material. DNA fragments were purified with the QIA extraction kit (Qiagen), ligated into the pGEM-T Easy vector (Promega), and electroporated into E. coli XL1-Blue cells. Transformants were selected on LB plates containing ampicillin, isopropyl-β-d-thiogalactopyranoside (IPTG) (0.5 mM), and X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) (50 μg ml⁻¹) to allow blue-white screening. The verified DNA inserts of positive clones were digested with either NdeI and EcoRI or NdeI and SalI and ligated into either pET28a(+) or pET30a(+) (Novagen) linearized with the corresponding enzymes to yield pET-nrdH, pET-nrdE, and pET-nrdF. The resulting constructs were introduced into E. coli XL1-Blue cells. The plasmid constructs were verified by sequencing and introduced into E. coli BL21(DE3) for protein expression. Cultures of E. coli BL21(DE3) containing pET-nrdH, pET-nrdE, and pET-nrdF as well as pOUA and pOUB, carrying genes encoding the S. aureus thioredoxin and thioredoxin reductase, were grown overnight at 37°C in LB medium containing 50 mM Tris-HCl (pH 8.0), phenylmethylsulfonyl fluoride (PMSF) (50 mM Tris-HCl (pH 8.0), phenylmethylsulfonyl fluoride (PMSF) (5 μg ml⁻¹) was discarded. The cell pellet was stored at −80°C.

Assay of ribonucleotide reductase activity. NR activity was assayed by the conversion of GDP to dGDP (42). Assay mixtures of 50 μl were incubated for 20 min at 30°C and contained 50 mM Tris-Cl (pH 7.6), 5 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.2 mM dATP, 0.56 mM [³²P]dCTP (28,000 cpm mmol⁻¹), 20 μg of NrdE, and 10 μg of NrdF. The amount of dCDP formed was determined by a standard method as previously described (42). One unit of enzyme corresponds to 1 nmol of dCDP formed min⁻¹, and the specific activity is expressed as units/mg of total protein.

Insulin, DTNB, and glutaredoxin assays. Insulin assays were performed according to a method described previously by Holmgren (14), DTNB (5.5-dithio-bis(2-nitrobenzoic acid)) assays were performed according to a method described previously by Jordan et al. (16), and GSH-disulfide oxidoreductase assays were performed according to a method described previously by Latham and Holmgren (25).

Bioinformatics analysis. Open reading frame (ORF) searches were performed with the National Center for Biotechnology Information (NCBI) ORF Finder server (http://www.ncbi.nlm.nih.gov/orf/). Sequence entries and primary analyses of DNA and protein sequences were performed with the Clone Manager 7 program (Scientific & Educational Software, Durham, NC). Primary sequences of S. aureus NrdH-like proteins were identified in databases of the University of Oklahoma’s Advanced Center for Genome Technology (http://www.genome.ou.edu), the Comprehensive Microbial Resource of the J. Craig Venter Institute (http://cmr.jcvi.org/cgi-scripts/CMR/CmiHomePage.cgi), the Staphylococcus aureus Sequencing Group at the Sanger Centre (http://www.sanger.ac.uk/sequencing/Staphylococcus/aureus/), and the NCBI Microbial Genomes server (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=microb) by use of the tblASTIn algorithm (1). Pairwise and multiple-sequence alignments were performed with the ClustalW program, version 1.84 (13); with the EMBO ClustalW2 server (http://www.ebi.ac.uk/Tools/clustalw2/index.html); and with the Network Protein Sequence Analysis server (http://pspa-pbil.ibcp.fr/cgi-bin/pnps_automat.pl?page=PNPS/pspa_clustal.html). Position-specific iterated (PSI) BLAST searches (1) were performed to find close homologs of S. aureus NrdH. The searches included three iterations on the UniProt database of proteins sequences (http://www.uniprot.org). A multiple-sequence alignment was built by using MUSICLE (8) with 200 homologous proteins found by PSI BLAST and the two Protein Data Bank (PDB) templates. The aligned sequences of the S. aureus, C. ammoniagenes, and E. coli NrdH proteins were extracted and used to build structure models of S. aureus NrdH. Phylogenetic and molecular evolutionary analyses were carried out using MEGA 4 (41).

Structure modeling. Appropriate three-dimensional (3D) templates suitable for the modeling of S. aureus NrdH were obtained from the FFA30 server (http://fas.jcrcf.edu/fas/fgfas/fgfas.pl) (34). The C. ammoniagenes NrdH X-ray structure (PDB accession number 1R7H) (39) was the best result, followed by the Brucella melitensis glutaredoxin nuclear magnetic resonance (NMR) structure (PDB accession number 2KHP) and the E. coli NrdH X-ray structure (PDB accession number 1H75) (40). The two X-ray structures were used to model the S. aureus NrdH structure, employing residues 2 to 77; two models were built, one based on a monomer template and the second based on a dimer template.

Model building and validation. The first model was based on the monomer-state X-ray structure of E. coli NrdH (PDB accession number 1H75) (40). The model was built using the SCWRL4 side-chain conformation prediction software (20). The sequence identity between the S. aureus NrdH and E. coli NrdH proteins is 22%. Since the alignment between the sequences of the two proteins contains only one gap, which is located prior to the second alpha helix (α2), the two structure models that the side chains only is not sufficient in this case. The models were further assessed by using the MolProbity server (http://molprobity.biochem.duke.edu/) (6, 23), which examines ψ and φ angles, Cβ deviations, atom clashes, and rotamers. The conservation score calculated by ConSurf (21), based on both the multiple-sequence alignment described above, was projected onto the two structure models in order to check whether the conserved amino acids are in the core of the protein structure and the variable amino acids are on the periphery. The three structures were superimposed by using the MultiProt server (http://bioinfo3d.cs.tau.ac.il/MultiProt/) (38). The root mean square deviation (RMSD) between S. aureus NrdH and C. ammoniagenes NrdH is 16.8%. Here the gap is located inside α2 of the template structure, so a method that models the side chains only is not sufficient in this case. The models were further assessed using the MOLPROBITY biochemistry database (http://molprobity.biochem.duke.edu) (6, 23), which examines ψ and Φ angles, Cβ deviations, atom clashes, and rotamers. The conservation score calculated by ConSurf (21), based on both the multiple-sequence alignment described above, was projected onto the two structure models in order to check whether the conserved amino acids are in the core of the protein structure and the variable amino acids are on the periphery. The three structures were superimposed by using the MultiProt server (http://bioinfo3d.cs.tau.ac.il/MultiProt/) (38). The root mean square deviation (RMSD) between S. aureus NrdH and C. ammoniagenes NrdH is 0.54 Å, and the RMSD between S. aureus NrdH and E. coli NrdH is 0.47 Å.

Nucleotide sequence accession number. The nucleotide sequence of the DNA region containing the S. aureus strain RN4220 nrdH gene has been deposited in the EMBL/GenBank/DDBJ database under accession no. AJ416621.

RESULTS AND DISCUSSION

Staphylococci contain an NrdH-like protein. We previously reported that S. aureus, unlike E. coli and L. lactis, lacks an nrdH gene in the class Ib RNR operon (26, 45). An identical operon structure is found in other staphylococci and in certain bacilli belonging to the Bacillus cereus group. We assumed that these bacteria, all of which contain a single aerobic class Ib RNR and lack a glutathione thioredox redox system, are likely to possess an NrdH redox protein. Initial attempts to identify an nrdH gene in S. aureus strains COL (11) and NCTC 8325 were performed in BLAST searches using the E. coli (16), L. lactis (17), and C. ammoniagenes (9) NrdH amino acid sequences as queries but were unsuccessful. A more refined PSI-BLAST search using a single L. lactis NrdH protein sequence as a query and an E value threshold of 0.02 led to the identification of a remote nrdH homolog, which contained a characteristic C-X-C-X-C redox motif. The nrdH-like gene is located between, and oriented in the opposite direction of, the cdBA genes.
encoding subunit I of the bd-type ubiquinol oxidase, and the ptsf gene, encoding phosphoenolpyruvate-dependent phosphotransferase, and is far removed from the nrdIEF gene cluster (25). A single promoter containing well-defined -10 and -35 recognition elements is positioned upstream of the nrdH structural gene (data not shown). We identified nrdH-like orthologs in the genomes of other Staphylococcus species, including S. aureus (NP_846437), respectively. For example, it shares 22% and 27% identities with E. coli NrdH and L. lactis NrdH, respectively, and 12% and 25% identities with E. coli glutaredoxin-1 (GrxA) and glutaredoxin-3 (GrxC), respectively. In the N-terminal portion of the protein, it contains a motif containing two cysteines, C-P-P-C, and a thioredoxin-like activity profile (39). Alignment of 15 NrdH-like ORFs extracted from genomes of several geographic groups of NrdH proteins shown are listed in Table S1 in the supplemental material.

The S. aureus NrdH-like protein has a molecular mass of ~9.2 kDa and a relatively low isoelectric point (3.95 to 4.10 in different strains). Comparative sequence analysis of S. aureus NrdH shows that it is related to the well-characterized NrdH redoxin (NrdH) and glutaredoxin (Grx) families of proteins. For example, it shares 22% and 27% identities with E. coli NrdH and L. lactis NrdH, respectively, and 12% and 25% identities with E. coli glutaredoxin-1 (GrxA) and glutaredoxin-3 (GrxC), respectively. In the N-terminal portion of the protein, it contains a motif containing two cysteines, C-P-P-C, and a thioredoxin-like activity profile (39). Alignment of 15 NrdH-like ORFs extracted from genomes of several geographic groups of NrdH proteins shown are listed in Table S1 in the supplemental material.

FIG. 1. ClustalW multiple-sequence alignment of NrdH and NrdH-like bacterial proteins. Experimentally characterized S. aureus (SAU), E. coli (ECO), and C. ammoniagenes (CAM) NrdH orthologs are indicated in white boldface in black boxes. Redox-active residues are shown in gray boxes. Identical residues are indicated by asterisks, highly similar residues are indicated by colons, and weakly similar residues are indicated by dots (all shown in boldface type). SEP, Staphylococcus epidermidis; SHA, S. haemolyticus; WALT, Staphylococcus warneri; BTA, B. anthracis; BWE, Bacillus weihenstephanensis; BCO, Bacillus coahuilensis; Bsp, Bacillus sp. NRRR B-14911; STY, S. Typhimurium; DZE, Dickeya zeae; AGF, Agrobacterium tumefaciens; BME, Brucella melitensis; BLO, Bifidobacterium longum; MTU, M. tuberculosis; AOD, Actinomyces odontolyticus; LAC, Lactococcus lactis; AOD, Actinomyces odontolyticus; CAM, C. amniogenes. GenBank accession numbers of the NrdH proteins shown are listed in Table S1 in the supplemental material.
NrdH proteins. Nevertheless, a comparison of the predicted secondary structure of *S. aureus* NrdH with that of *E. coli* NrdH shows that they possess very similar secondary structures (data not shown). In the following sections, we refer to SACOL1093 (NrdH-like protein in *S. aureus* strain COL) and its homologs as NrdH proteins. The phylogeny of NrdH protein sequences confirms that the staphylococcal and bacillus NrdH proteins are separated from the canonical NrdH redoxins as well as from the glutaredoxins (39, 44) (Fig. 2). NrdH-like proteins have also been reported for members of the Archaea (3). The *Methanococcus jannaschii* redoxin Mj0307 (3) shares about 20% sequence identity with the mesophilic NrdH redoxins and is similar in its sequence to glutaredoxins yet is structurally different from *E. coli* NrdH and, like *S. aureus* NrdH, lacks the C-terminal motif.

**S. aureus** NrdH structure prediction. Homology modeling of the *S. aureus* NrdH structure, based on the known *E. coli* NrdH 3D structure, shows that their structures are closely related: each possesses a four-stranded β-sheet and three flanking helices typical of the thioredoxin superfamily (Fig. 3). Superposition of the *S. aureus* NrdH and *E. coli* NrdH structures resulted in an RMSD of 0.47 Å. The predicted *S. aureus* NrdH structure has an active site and surface topology similar to those of the *E. coli* redoxin but lacks those residues in glutaredoxins that are responsible for binding glutathione. NrdH proteins contain a highly conserved C-terminal sequence motif,
FIG. 3. Homology structural model of *S. aureus* NrdH. (A and B) *S. aureus* NrdH model structure (see Materials and Methods) (A) and *E. coli* NrdH X-ray structure (PDB accession number 1H75) (B) (40) presented in cartoons. The two cysteines of the CXXC redox-active site are shown as sticks and are labeled. The residues of the loop connecting β4 to α3 are shown as sticks and are colored blue (A) and cyan (B). (C) Superposition of the two structures performed by using Multiprot (38). The RMSD between the two structures is 0.47 Å.

61WSGFRP(D/E)67, which in the *E. coli* NrdH structure links β4 to the α3 helix and creates an intricate network of H bonds involving the residue side chains and two water molecules. A salt bridge occurs between Arg65 and Asp67 (40). Curiously, the corresponding region in *S. aureus* NrdH 61MYHV DLD67 is quite different. Plausibly, it performs the same function as in *E. coli* NrdH in stabilizing the loop connecting β4 to α3. Note that in *S. aureus* NrdH, Asp65 replaces Arg65 in *E. coli* NrdH, which eliminates the salt bridge between Arg65 and Asp67. However, a new salt bridge may be formed between His63 and Asp65. The rim of the hydrophobic pocket in *E. coli* NrdH contains three arginines (Arg8, Arg44, and Arg49), which create a positively charged surface around the cavity; in the *S. aureus* NrdH structural model, the corresponding residues are Glu, Ile, and Phe. In contrast to *E. coli* NrdH, which has a deep hydrophobic pocket next to the active site where thioredoxin reductase is proposed to bind (40), the *S. aureus* NrdH cavity is shallower. The residues in *S. aureus* NrdH that correspond to those in *E. coli* NrdH that form the bottom of the pocket and surround it are similarly hydrophobic. However, the *E. coli* and *S. aureus* NrdH proteins differ noticeably in their electrostatic and hydrophobic profiles (see Fig. S2 in the supplemental material).

*S. aureus* nrdH is not essential for aerobic and anaerobic growth. Preliminary experiments showed that *S. aureus* nrdH is transcribed under aerobic and anaerobic conditions (data not shown). To assess whether *S. aureus* nrdH is essential for growth, an nrdH deletion mutant was created by using a gene replacement strategy (see Materials and Methods). Plasmid pUP-Km-DW (Table 1), a derivative of pMUTIN-4 (47), was used to replace the chromosomal nrdH gene with the kanamycin resistance cassette. Strain SH1000 and the ΔnrdH deletion mutant were grown in liquid in TSB medium under aerobic and anaerobic conditions. No discernible difference was observed in the rate and extent of growth between the parent and mutant strains, establishing that NrdH is not essential under either growth condition. For aerobic growth, the generation time of both strains was ~40 min, and maximal growth was reached, as judged by the absorbance, at the stationary phase (optical density at 600 nm [OD600] of ~8). For anaerobic growth, the generation time of the two strains was ~65 min, and maximal growth was reached in the stationary phase (OD600 of 1.8 to 1.9). These results establish that *S. aureus* NrdH is not essential for growth under the conditions employed.

To assess whether NrdH plays a role in protection against oxidative stress, cultures of the wild type and the ΔnrdH mutant were grown in TSB medium and treated with hydrogen peroxide at concentrations of 0 to 25 mM, and the effect on growth was monitored as described previously (48). No significant difference was found for the growths of the parent and mutant strains (data not shown). Similarly, no significant difference was found when cells were treated with 2 to 8 mM diamide, a thiol-specific oxidant that promotes disulfide bond formation (data not shown). NrdH therefore does not appear to have a role in coping with oxidative stress under the conditions employed.

NrdH reduces protein disulfides. To assess whether *S. aureus* NrdH possesses thiol-disulfide redox activity, we tested the ability of recombinant NrdH to reduce protein disulfides. Insulin was used as the disulfide substrate in an assay previously developed for measuring thioredoxin redox activity (14). The chemical or enzymatic reduction of disulfide bonds results in the precipitation of insulin chains, which can be monitored by an increase in turbidity. *E. coli* thioredoxin was used as a positive control; DTT (dithiothreitol) at a concentration of 1 mM served as a negative control. Figure 4A shows that in the presence of the artificial disulfide reductant DTT (1 mM), the addition of NrdH to the standard reaction mix caused a marked increase in turbidity as measured by determining the
E. coli NrdH possesses GSH-disulfide oxidoreductase activity. S. aureus lacks glutathione, we tested whether S. aureus NrdH possesses GSH-disulfide oxidoreductase activity. The assay was performed in the presence of 1 mM DTT and increasing concentrations of 2 μM (■), 4 μM (▲), and 8 μM (●) the NrdH protein (A) and in the presence of 2 μM (○), 4 μM (△), and 8 μM (□) TrxA (B). Controls were no DTT added (×), no NrdH added (●), and no TrxA added (○).

**FIG. 4.** S. aureus NrdH and TrxA reduction of insulin disulfides. Turbidity increased with increasing NrdH concentrations, 2 μM and 8 μM, and time of incubation. Figure 4B shows that the addition of 2 μM and 8 μM E. coli thioredoxin to the standard reaction mix caused a similar increase in turbidity, which further increased with time. In both cases, the reduction of insulin with 1 mM DTT alone is extremely low. The results demonstrate that S. aureus NrdH as is effective, if not more so, as E. coli thioredoxin in reducing insulin disulfides.

We next tested the ability of the S. aureus NrdH protein to transfer electrons from S. aureus thioredoxin reductase to reduce DTNB. Control reactions were performed with S. aureus and E. coli thioredoxin reductase. Reactions were carried out in microplates as described in Materials and Methods. S. aureus NrdH was somewhat less effective in the reduction of DTNB than its cognate thioredoxin but was more effective than E. coli thioredoxin. Reduction rates measured by the ΔAbs/min were 0.020 for S. aureus NrdH, 0.033 for S. aureus thioredoxin, and 0.013 for E. coli thioredoxin.

Although S. aureus lacks glutathione, we tested whether S. aureus NrdH possesses GSH-disulfide oxidoreductase activity. The experimental system employs NADPH as the source of reducing power, reduced glutathione, yeast glutathione reductase, and 2-hydroxyethyl disulfide (HED) as substrates (see Materials and Methods). No significant consumption of NADPH occurred upon the addition of NrdH to the standard reaction mix, as judged by the lack of a change in the OD_{600nm} indicating that NrdH, like thioredoxin, lacks GSH-disulfide oxidoreductase activity (data not shown).

**FIG. 5.** Effect of DTT on stimulating NrdH-dependent S. aureus NrdEF ribonucleotide reductase activity. NrdE (20 μg) and NrdF (10 μg) were incubated in the standard assay (see Materials and Methods) in the presence of 4.5 μg of the NrdH protein (■) or in the absence of the NrdH protein (○).

NrdH is a hydrogen donor for the class Ib ribonucleotide reductase. S. aureus (26) and many other Gram-positive bacteria, including *C. ammonium* (44), *Mycobacterium tuberculosis* (7), and *B. subtilis* (37), contain a single aerobic class Ib RNR. Given that the S. aureus class Ib RNR is essential for aerobic growth (26) and lacks a glutathione-based redox system (27), we supposed that NrdH may play an important role in growth as a hydrogen donor for the class Ib RNR. To assess whether the S. aureus NrdH protein can function as an electron donor for the class Ib NrdEF RNR, we employed an *in vitro* assay to monitor the conversion of labeled CDP to dCDP (see Materials and Methods). His_{6}-tagged S. aureus NrdE and NrdF proteins were expressed in *E. coli* and affinity purified on a nickel-chelating column (see Materials and Methods). The conversion of CDP to dCDP was dependent on the NrdE and NrdF proteins and a hydrogen donor system. Figure 5 shows NrdEF activity as a function of the DTT concentration in the standard reaction mixture with (top curve) and without (bottom curve) NrdH. In the presence of NrdH, activity was maximal at 2 mM DTT, corresponding to a level of stimulation about 2.5-fold higher that that without NrdH. The data have been adjusted to deduct the residual activity found in the absence of added DTT, which is presumably due to the presence of trace amounts of DTT in the NrdEF and NrdH protein preparations, which is difficult to remove even after exhaustive dialysis of the protein preparations. At higher DTT concentrations the relative stimulation decreases as the DTT contribution to activity increases. Thus, NrdH can serve as an electron donor for the class Ib RNR. As a control, hydroxyurea, an inhibitor of class I RNRs, abolished RNR activity in a concentration-dependent way (data not shown).

To establish that S. aureus NrdH is able to reduce NrdEF via thioredoxin reductase, we replaced DTT in the activity assay mixture with NADPH and the cognate thioredoxin reductase.
Table 2 shows that NrdH serves as a reductant for the class Ib RNR with NADPH and thioredoxin reductase as the hydrogen donor system. Reduction by NrdH was almost as effective as that by thioredoxin in transferring the reducing power from NADPH via thioredoxin reductase. These results are consistent with in vitro studies of the E. coli, L. lactis, S. Typhimurium, and C. ammoniagenes class Ib RNR systems and more-recent in vivo studies of E. coli by Gon and coworkers, which showed that NrdH specifically reduces class Ib and is recycled by thioredoxin reductase (12). Although S. aureus lacks glutathione, we asked whether NrdH was unable to mediate the transfer of electrons from NADPH to NrdEF when glutathione and glutathione reductase were used in place of NrdH and thioredoxin reductase. The results (Table 2) show that no activity was detected.

The effects of the allosteric effectors ATP and dATP on the class Ib RNR activity were examined. dATP was found to strongly stimulate CDP reduction with an optimal activity at 2.0 mM, whereas ATP had little or no effect on activity (Fig. 6A). ATP had little or no effect on activity (Fig. 6A). When ATP was added to the standard reaction mixture containing 2.0 mM dATP, the reaction rate decreased with increasing ATP concentrations from 0 to 10 mM (Fig. 6B). These finding are similar to those reported previously for the E. coli and L. lactis NrdEF enzymes (17, 18).

Concluding remarks. The existence of multiple hydrogen donor systems in bacterial RNRs is well documented (29). S. aureus contains two well-characterized thiol redox systems, NrdH-thioredoxin reductase (NrdH/TrxB) (this work) and thioredoxin-thioredoxin reductase (TrxA/TrxB) (46). However, S. aureus and most other Gram-positive bacteria lack glutathione, the most common thiol in nature. Bacillithiol, a recently discovered low-molecular-weight antioxidant thiol abundant in Bacillus and S. aureus species, was proposed to serve as a substitute for glutathione in these bacteria (28). In this paper we show that S. aureus NrdH is an efficient reductant in vitro of disulfide bonds in low-molecular-weight substrates and proteins. Its activity in transferring electrons from NADPH via thioredoxin reductase to these substrates is comparable in efficiency to that of the homologous thioredoxin and thioredoxin reductase. Moreover, the S. aureus NrdH and thioredoxin proteins were equally as efficient as reductants of NrdEF. Thus, NrdH or thioredoxin can potentially function, with thioredoxin reductase, as the immediate reductant of the class Ib NrdEF RNR. These findings are consistent with the finding that S. aureus thioredoxin reductase is essential for aerobic growth (46). Since we show here that NrdH is dispensable for aerobic (and anaerobic) growth, thioredoxin is likely to be primarily responsible for the reduction of the class Ib RNR. Our conclusions are consistent with data from a recent study by Chaudhuri et al. (4), who performed a comprehensive mutational analysis of S. aureus SH1000 (the same strain used in this study) and showed that both thioredoxin and thioredoxin reductase are essential genes. Similarly, thioredoxin was found to be essential in Bacillus subtilis, which, like S. aureus, contains a class Ib RNR and lacks a glutathione redox system, based on the inability to isolate an insertional trxA mutant (36).
not expressed in sufficient amounts to support growth when the class Ia RNR is inactive. However, when NrdEF is overexpressed (e.g., from a plasmid), it can support growth (12). In *in vitro* studies of *E. coli* showed that NrdH reduces NrdEF, whereas thioredoxin (TrxA) does not (16). Because *S. aureus* class Ib RNR, whereas thioredoxin (TrxA) does not (16). Because *S. aureus* and comment.

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**REFERENCES**


