NOTES

A Novel Nitroreductase of *Staphylococcus aureus* with *S*-Nitrosoglutathione Reductase Activity[▽][†]

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In this report we show that inactivation of the putative nitroreductase SA0UHSC_00833 (*ntrA*) increases the sensitivity of *Staphylococcus aureus* to *S*-nitrosoglutathione (GSNO) and augments its resistance to nitrofurans. *S. aureus* NtrA is a bifunctional enzyme that exhibits nitroreductase and GSNO reductase activity. A phylogenetic analysis suggests that NtrA is a member of a novel family of nitroreductases that seems to play a dual role in vivo, promoting nitrofuran activation and protecting the cell against transnitrosylation.

Staphylococcus aureus is a gram-positive pathogen responsible for a large number of human infections that range from mild to potentially lethal systemic infections. The rapidly increasing incidence of methicillin-resistant *S. aureus* infections, particularly among human immunodeficiency virus-infected and AIDS patients (1), reveals that the antibiotic of choice for the treatment of *S. aureus* is becoming ineffective and shows the need for using alternative compounds. *Staphylococcus* strains are sensitive to nitrofuran derivatives such as nitrofurazone and nitrofurantoin, which are utilized in the treatment of burns, skin grafts, and genitourinary infections (2). The action of nitrofurans is dependent on the presence of specific microbial enzymes, the nitroreductases, which catalyze the reduction of the drug, a step that is essential for its activation. The activation of nitroaromatic compounds by bacterial nitroreductases is also used as a cancer therapy, since the cytotoxic hydroxylamine derivative compounds are able to destroy tumors (5).

In microorganisms subjected to nitrosative stress, *S*-nitrosoglutathione (GSNO) is formed by reaction of NO with the intracellular glutathione. The GSNO formed reacts with thiol-containing proteins promoting thiol nitrosation, which modifies the function of proteins that are essential to many cellular processes. To control the level of *S*-nitrosylated proteins, organisms use GSNO reductases, namely, the ubiquitous glutathione-dependent formaldehyde dehydrogenase, also known as class III alcohol dehydrogenase (6). However, GSNO is also a NADPH-dependent oxidizing substrate of other enzymes such as the thioredoxin system, glutathione peroxidase, γ-glutamyl transpeptidase, and xanthine oxidase, indicating that GSNO reductase activity is frequently associated with other enzymatic activities (3, 4, 7, 10).

Our microarray studies revealed that the staphylococcal gene SA0UHSC_00833 of *S. aureus* NCTC 8325 encoding a putative nitroreductase is induced by GSNO. Hence, we have analyzed the in vivo role of this protein in the metabolism of GSNO and nitrofurans and performed biochemical characterization of the recombinant protein SA0UHSC_00833 (named NtrA).

S. aureus NtrA is involved in GSNO metabolism. In a DNA microarray study of *S. aureus* NCTC 8325, we detected that GSNO significantly upregulates the SA0UHSC_00833 gene (herein named *ntrA*), which encodes a putative nitroreductase (our unpublished data). To perform quantitative real-time reverse transcription-PCR analysis, cells of *S. aureus* NCTC 8325 were grown in LB medium to an optical density at 600 nm of 0.4 and were treated for 10 min with GSNO (150 μM). The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Tables S1 and S2 in the supplemental material. Real-time PCR experiments were performed with a LightCycler FastStart DNA Master SYBR green I kit (Roche Applied Science), using 400 ng of cDNA prepared from total RNA and 0.5 μM of primers SANrEco and SaNrNhe for the SA0UHSC_00833 (*ntrA*) gene and primers 16S_Fwd and 16S_Rev for *S. aureus* 16S rRNA. The results confirm that GSNO causes a strong (13.4- ± 3.8-fold) transcriptional induction of the *ntrA* gene. The expression of SACOL0874, a homolog gene in *S. aureus* COL, was also reported to be induced by nitrosative stress conditions (8). Since these results suggest the involvement of the *ntrA* gene in nitrosative metabolism, a strain defective in the gene was constructed and analyzed. For construction of the mutant, the *S. aureus* NCTC 8325 SA0UHSC_00833 (*ntrA*) gene was PCR amplified using SA0833MutEco and SA0833MutBam oligonucleotides, ligated

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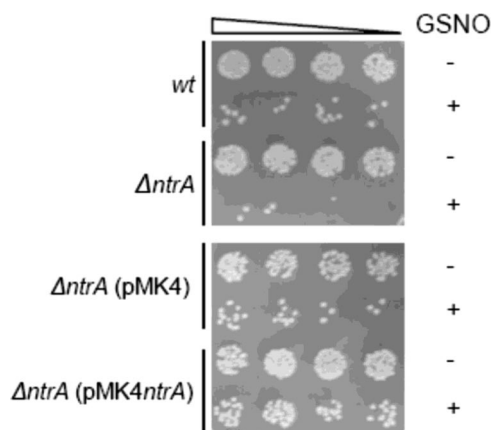
FIG. 1. Sensitivity to GSNO of the *S. aureus* Δ*ntrA* mutant. *S. aureus* wild type (wt), the Δ*ntrA* strain, and Δ*ntrA* strains harboring pMK4*ntrA* or the vector alone were grown in LB medium and treated with 1 mM of GSNO (+) or left untreated (−). Sensitivity tests were assayed after 4 h of GSNO treatment by plating on agar serial dilutions of the cultures.

to pSP64D-E (pSP64-SA0833), electroporated into *S. aureus* RN4220, and selected on tryptone soy agar plates containing erythromycin (10 μg/ml). The correct integration of pSP64-SA0833 into the chromosome of *S. aureus* was confirmed by PCR, and the generated mutant strain was designated LMSA833. For the GSNO sensitivity tests, the wild-type strain and LMSA833 (the Δ*ntrA* mutant) were grown overnight in tryptone soy broth (TSB), inoculated in fresh LB medium, and exposed for 1 h to GSNO (100 μM). The cultures were treated for another 4 h with 1 mM of GSNO, and serial dilutions were spotted onto tryptone soy agar plates. After overnight incubation, the plates revealed that the Δ*ntrA* mutant exhibited a higher degree of GSNO inhibition than the parental strain. Furthermore, expression of NtrA from the pMK4 plasmid restored the wild-type phenotype (Fig. 1). Nevertheless, we did not observe enhanced susceptibility of the *ntrA* mutant when using NO gas (data not shown).

The *S. aureus ntrA* mutant is more resistant to nitrofurans. Since SA0UHSC_00833 is annotated as a putative nitroreductase, its role in the metabolism of nitrofurans was also examined. To this end, overnight cultures of the *S. aureus* wild type (RN4220) and the Δ*ntrA* mutant (LMSA833) grown in TSB were used to inoculate fresh LB medium supplemented with nitrofurantoin (20 μg/ml) or nitrofurazone (25 μg/ml). In the absence of antibiotics, the wild-type and mutant strains displayed similar growth kinetics. After the addition of nitrofurans, the growth of both strains was immediately inhibited. Whereas after 22 h the wild type showed limited recovery (recovery values of 17% and 9% for nitrofurantoin and nitrofurazone, respectively), the mutant strain had resumed growth at a rate similar to that of the untreated culture, exhibiting a recovery of 67% for cells treated with nitrofurantoin and of 71% for cells submitted to nitrofurazone. Furthermore, cells of the *ntrA* mutant strain expressing NtrA from a multicopy plasmid bearing only the *ntrA* gene regained the sensitivity to nitrofurans with a phenotype similar to the phenotype observed for the wild-type strain (data not shown). In conclusion, the presence of an active *ntrA* gene product contributes to the sensitivity of *S. aureus* to nitrofurans.

Mutation of *ntrA* decreases the nitroreductase activity of *S. aureus*. The activity of cell extracts of the *S. aureus* wild type and the *ntrA* mutant strain were analyzed by following the reduction of nitrofurantoin and nitrofurazone through the use of NADH or NADPH as an electron donor. To this end, cells from TSB overnight cultures of the wild type and the Δ*ntrA* mutant (LMSA833) were harvested, resuspended in cold 20 mM Tris-HCl buffer (buffer A) (pH 7.6), and incubated with lysostaphin (50 μg/ml) at 37°C for 15 min. Following the addition of DNase (50 μg/ml), cells were centrifuged (13,000 × *g*) for 30 min at 4°C. The enzymatic assays were recorded using a Shimadzu UV-1700 spectrophotometer (at 25°C) following the decrease of the nitrofuran absorbance (for nitrofurazone, $\varepsilon_{400} = 12{,}960$ M^{-1} cm^{-1}; for nitrofurantoin, $\varepsilon_{420} = 12{,}000$ M^{-1} cm^{-1}). The mixture contained buffer A, nitrofuran, NADPH, or NADH (100 μM), and the reaction was initiated by the addition of the cell extract. The NADPH-dependent activity was found to be higher than NADH-dependent activity, and the higher activity values were measured when using nitrofurantoin. Inactivation of the *ntrA* gene of *S. aureus* led to a significant decrease in the nitrofurantoin and nitrofurazone reductase activity values (Table 1), which correlates with the increase in the nitrofuran resistance observed in the *ntrA* mutant strain.

***S. aureus* NtrA protein reduces GSNO and nitrofurans.** To further characterize the function of the *S. aureus* NtrA, the protein was produced and characterized. Hence, the *S. aureus* NCTC 8325 SA0UHSC_00833 (*ntrA*) gene was amplified using two oligonucleotides, SA0833NheIEx and SA0833EcoRIEx (see Table S2 in the supplemental material), and cloned into pET28a(+), yielding pET28NtrA, which was transformed in *Escherichia coli* BL21-Gold(DE3). Cells were grown in LB medium with 30 μg of kanamycin/ml to an optical density at 600 nm of 0.3 and induced for 7 h with isopropyl-ß-D-thiogalactopyranoside (IPTG) (500 μM). Since the NtrA protein produced contain a His tag, the soluble extract was loaded onto a HisTrap Hp column (GE Healthcare). Elution with ∼200 mM imidazole yielded a pure fraction of NtrA, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which had an apparent molecular mass of ∼21 kDa. The GSNO reductase activity of *S. aureus* NtrA was measured in buffer A–*S. aureus* NtrA protein (0.6 μM)–NADPH (or NADH) (250 μM)–GSNO (50 to 400 μM). The reaction was initiated by the addition of GSNO and monitored following the oxidation of NAD(P)H ($\varepsilon_{320} = 6{,}220$ M^{-1} cm^{-1}). Activity values were corrected, taking into account the reaction of GSNO with NAD(P)H. The kinetic data were fitted to the Michaelis-Menten equation by the use of SigmaPlot software. All values shown here represent the average results of at least

TABLE 1. Nitroreductase activity assayed in cell extracts of *S. aureus* wild-type and *ntrA* mutant (Δ*ntrA*) strains

Strain	Nitroreductase activity (nmol · min^{-1} · mg^{-1} protein)[a]			
	Nitrofurantoin		Nitrofurazone	
	NADPH	NADH	NADPH	NADH
Wild type	9.5 ± 0.7	4.7 ± 0.5	4.8 ± 0.4	2.8 ± 0.4
Δ*ntrA*	5.6 ± 0.4	3.1 ± 0.2	2.7 ± 0.2	1.9 ± 0.3

[a] Values are means ± standard errors.

TABLE 2. GSNO reductase and nitroreductase activity of
S. aureus NtrA

Enzyme	K_m (µM)	V_{max} (µmol · min^{-1} · mg^{-1})	k_{cat} (min^{-1})
GSNO	180.6 ± 43.4	1.5 ± 0.2	1.2
Nitrofurantoin	18.8 ± 4.8	13.7 ± 1.5	22.8
Nitrofurazone	25.7 ± 5.8	15.3 ± 1.6	25.5

two assays. The *S. aureus* NtrA enzyme was shown to be specific for GSNO, since no activity was seen for NO, *S*-nitrosocysteine, or *S*-nitrosohomocysteine. Kinetic analysis of the *S. aureus* NtrA gave a K_m value (pH = 7.6) for GSNO of ~181 µM (Table 2), a value which is lower than that measured for *E. coli* glutathione-dependent formaldehyde dehydrogenase (K_m ~ 700 µM [pH = 7]) (6), thus indicating a higher affinity of NtrA for this substrate. The nitroreductase activity of the purified *S. aureus* NtrA was also determined using nitrofurantoin and nitrofurazone (5 to 50 µM) as substrates and, as electron donors, NADPH or NADH (250 µM), which were used to initiate the reaction. Since *S. aureus* NtrA (0.6 µM) had previously been incubated with flavin mononucleotide or flavin adenine dinucleotide (5 µM), all activities were corrected by taking into account the chemical reduction of the nitrofuran compound generated by the same amount of flavin utilized in the protein mix-
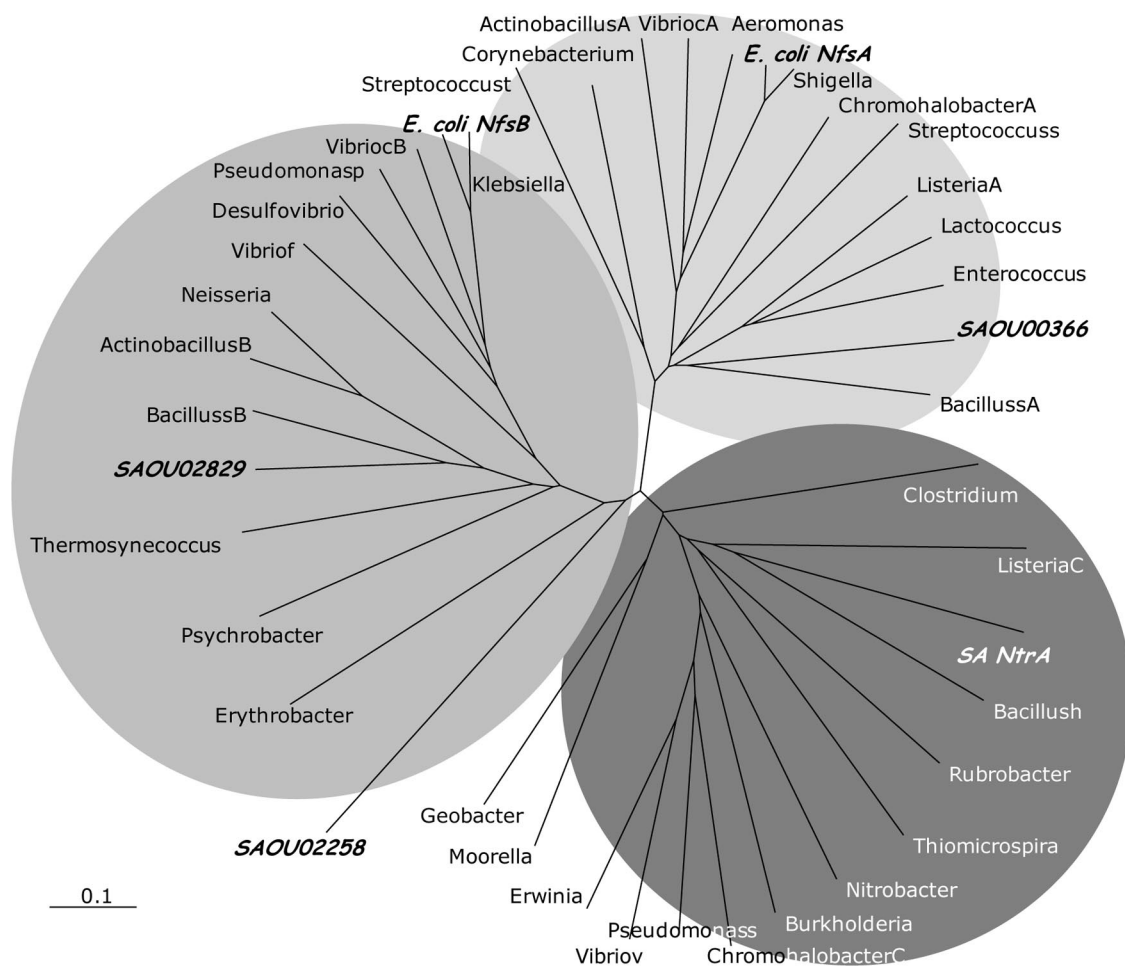

FIG. 2. Dendrogram of representative nitroreductases families. Organisms and protein sequence gi numbers corresponding to each abbreviation are as follows: ActinobacillusA (152979535) and ActinobacillusB (152979206), *Actinobacillus succinogenes*; Aeromonas, *Aeromonas salmonicida* (145298746); Bacillush, *Bacillus halodurans* (15612866); BacillussA (16080862) and BacillussB (16077850), *Bacillus subtilis*; Burkholderia, *Burkholderia thailandensis* (83716954); ChromohalobacterA (92112337) and ChromohalobacterC (92113480); *Chromohalobacter salexigens*; Corynebacterium, *Corynebacterium jeikeium* (68536654); Clostridium, *Clostridium difficile* (126700824); Desulfovibrio, *Desulfovibrio desulfuricans* G20 (78355133); *E. coli* NfsA, *E. coli* K-12 b0851 (16128819); *E. coli* NfsB, *E. coli* K-12 b0578 (16128561); Enterococcus, *Enterococcus faecalis* (29375756); Erythrobacter, *Erythrobacter litoraliss* (85374565); Erwinia, *Erwinia carotovora* (50121266); Geobacter, *Geobacter uraniumreducens* (148263412); Klebsiella, *Klebsiella pneumoniae* (152969124); Lactococcus, *Lactococcus lactis* (125623424); ListeriaA (16799400) and ListeriaC (16799563), *Listeria innocua*; Moorella, *Moorella thermoacetica* (83590668); Neisseria, *Neisseria gonorrhoeae* (59800832); Nitrobacter, *Nitrobacter winogradskyi* (75676226); Pseudomonasp, *Pseudomonas putida* (148548471); Pseudomonass, *Pseudomonas stutzeri* (146282796); Psychrobacter, *Psychrobacter* sp. (148653797); Rubrobacter, *Rubrobacter xylanophilus* (108804130); SA0U02258, *S. aureus* SA0UHSC_02258 (88195929); SA0U00366, *S. aureus* SA0UHSC_00366 (88194164); SA0U02829, *S. aureus* SA0UHSC_002829 (88196463); SA NtrA, *S. aureus* SA0UHSC_00833 (88194591); Shigella, *Shigella flexneri* (24112221); Streptococcuss, *Streptococcus sanguinis* (125718603); Streptococcust, *Streptococcus thermophilus* (55821160); Thermosynecoccus, *Thermosynechococcus elongatus* (22299744); Thiomicrospira, *Thiomicrospira crunogena* (78484399); VibriocA (15640734) and VibriocB (15601395), *Vibrio cholerae*; Vibriof, *Vibrio fischeri* (59713296); Vibriov, *Vibrio vulnificus* (27366397).

ture. We observed that whereas *S. aureus* NtrA incubated with flavin mononucleotide exhibited nitroreductase activity, no significant activity was measured when flavin adenine dinucleotide was incorporated into *S. aureus* NtrA. As observed for the GSNO reductase activity of *S. aureus* NtrA, the nitroreductase activities seen when using NADH or NADPH as a source of reducing equivalents were of the same order of magnitude (data not shown). The NADPH-dependent nitrofuran activity of *S. aureus* NtrA (Table 2) is within the range of values usually observed. In particular, the only nitroreductase from *S. aureus* that has been studied so far, the SA0UHSC_00366 protein (named NfrA), exhibits nitrofurazone- and nitrofurantoin-specific activities of 20 and 15 μmol·min^{-1}·mg^{-1}, respectively (12), and the *E. coli* NsfA nitroreductase has a specific activity of ~80 μmol·min^{-1}·mg^{-1} (9).

The bifunctional *S. aureus* NtrA belongs to a novel family of nitroreductases. A search in the genome of *S. aureus* NCTC 8325 revealed that in addition to SA0UHSC_00833 (NtrA), three other genes encoding putative nitroreductases are present, namely, SA0UHSC_00366 (NfrA), SA0UHSC_02829, and SA0UHSC_02258. The gene products of the four *S. aureus* nitroreductases share low levels of sequence identities, which range between 4% and 11%. Since the *E. coli* nitroreductases are well characterized (11), we performed an amino acid sequence alignment with these enzymes which revealed that SA0UHSC_00366 (NfrA) shares the highest percentage of identity with *E. coli* b0851 (NfsA) and SA0UHSC_02829 with *E. coli* b0578 (NfsB), whereas SA0UHSC_00833 (NtrA) and SA0UHSC_02258 show less than 10% identity with the two *E. coli* nitroreductases. A search in the completed microbial genome databases for homologs of the *S. aureus* nitroreductase NtrA yielded several gene products with percentages of sequence identity and similarity that range between 10% and 36%. A dendrogram was constructed based on the alignment of selected amino acid sequences of proteins found to be similar to SA0UHSC_00366 (NfrA), SA0UHSC_00833 (NtrA), SA0UHSC_02258, SA0UHSC_02829, *E. coli* NfsA, and *E. coli* NfsB (identity between the selected sequences ranges between 4 and 90%) (Fig. 2). The dendrogram suggests that there are three main groups. The first contains *E. coli* NfsB, SA0UHSC_02829, and SA0UHSC_02258, with SA0UHSC_02258 being the most distantly related member of this group, and the second includes *E. coli* NfsA and SA0UHSC_00366 (NfrA). The NtrA protein (SA0UHSC_00833) is located in a third independent group that does not contain any of the *E. coli* or *S. aureus* nitroreductases, thus suggesting that *S. aureus* NtrA is a member of a novel family of nitroreductases. Interestingly, the genome region comparison of the *ntrA* loci among the nine completely sequenced *S. aureus* genomes showed that, in all cases, the *ntrA* gene is flanked upstream by a gene encoding a protein similar to a 3-dehydroquinate dehydratase and downstream by a thioredoxin-encoding gene that is transcribed from the opposite strand.

In conclusion, we have shown that *S. aureus* NtrA is a bifunctional enzyme able to catalyze the reduction of GSNO and nitrofurans. Although the existence of a single protein that combines two activities seems to be a common feature among the GSNO reductases known so far, *S. aureus* NtrA is the first staphylococci nitroreductase shown to metabolize toxic nitrosothiols.

Even though the presence of NtrA contributes to the activation of nitrofurans, in the absence of these antibiotics NtrA acts as a defense system that allows the decomposition of endogenously formed GSNO, which occurs when *S. aureus* is exposed to nitrosative stress, thus avoiding the harmful effects caused by transnitrosylation reactions.

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