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Widespread Distribution in Pathogenic Bacteria of Di-Iron Proteins That Repair Oxidative and Nitrosative Damage to Iron-Sulfur Centers[▽]†

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Expression of two genes of unknown function, *Staphylococcus aureus scdA* and *Neisseria gonorrhoeae dnrN*, is induced by exposure to oxidative or nitrosative stress. We show that DnrN and ScdA are di-iron proteins that protect their hosts from damage caused by exposure to nitric oxide and to hydrogen peroxide. Loss of FNR-dependent activation of *aniA* expression and NsrR-dependent repression of *norB* and *dnrN* expression on exposure to NO was restored in the gonococcal parent strain but not in a *dnrN* mutant, suggesting that DnrN is necessary for the repair of NO damage to the gonococcal transcription factors, FNR and NsrR. Restoration of aconitase activity destroyed by exposure of *S. aureus* to NO or H_2O_2 required a functional *scdA* gene. Electron paramagnetic resonance spectra of recombinant ScdA purified from *Escherichia coli* confirmed the presence of a di-iron center. The recombinant *scdA* plasmid, but not recombinant plasmids encoding the complete *Escherichia coli sufABCDSE* or *iscRSUAhscBAfdx* operons, complemented repair defects of an *E. coli ytfE* mutant. Analysis of the protein sequence database revealed the importance of the two proteins based on the widespread distribution of highly conserved homologues in both gram-positive and gram-negative bacteria that are human pathogens. We provide in vivo and in vitro evidence that Fe-S clusters damaged by exposure to NO and H_2O_2 can be repaired by this new protein family, for which we propose the name *r*epair of *i*ron *c*enters, or RIC, proteins.

Neutrophils and macrophages of the mammalian immune system produce reactive oxygen and reactive nitrogen species that have important roles in killing pathogenic bacteria by damaging such cellular components as DNA, lipids, and proteins. Particularly vulnerable to inactivation are iron-sulfur (Fe-S) proteins, which were among the first catalysts used by nature (19). They participate in numerous cellular processes in virtually all organisms where they fulfill crucial redox, catalytic, and regulatory functions (1, 21, 28). Specialized systems have evolved that facilitate the assembly and insertion of Fe-S clusters into proteins, namely, the products of *isc*, *suf*, and *csd* operons (12, 13, 21). Analysis of bacterial genomes shows that one or more of these systems can be present in any organism for the in vivo maturation of Fe-S proteins. The *isc* operon encodes several proteins that are necessary for de novo synthesis, and at least one of them, IscS, is proposed to be required for cluster repair (9). The Suf system sustains Fe-S cluster biogenesis during iron starvation and oxidative stress (9, 38, 40), and CSD is proposed to act as a sulfur-generating system (34). Despite their established roles in pathogen survival, little is known about how oxidative and nitrosative damage to Fe-S clusters is repaired since so far only IscS is proposed to have such a function (9, 46, 58).

Transcriptomic studies have shown that nitrosative stress conditions elicit increased expression of not only the *isc* and *suf* operons but also various genes of known and unknown function (6, 25, 37, 43, 44, 47). The products of some of these genes are required to detoxify the reactive nitrogen species and are under the control of iron-sulfur regulators. For example, the *hmpA* gene present in various bacteria encodes an enzyme that catalyzes the oxidation of NO to nitrate in aerobic cultures or the reduction to nitrous oxide during anaerobic growth (14, 15, 29, 42). In *Escherichia coli*, expression of *hmpA* is repressed by FNR, the regulator of fumarate and nitrate reduction, which contains an $[4Fe\text{-}4S]^{+2/+1}$ iron-sulfur center that is essential for the binding of FNR to its DNA binding site. FNR, originally identified as an oxygen-sensitive transcription regulator, is also inactivated on exposure to nitric oxide, providing a mechanism by which FNR-repressed genes respond to nitrosative stress (7, 39). Similarly, the repressor activity of NsrR, which from sequence analysis is assumed to contain an [2Fe-2S] iron-sulfur center, is inactivated on exposure to nitric oxide (2, 10, 41). There is overlap between the biological responses to oxidative stress caused by exposure to hydrogen peroxide and to nitrosative stress (4, 18, 48, 59, 60). This overlap in-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
E. coli strains		
BL21 Gold(DE3)	Protein expression cells	Stratagene
K-12 ATCC 23716	Parental strain	Laboratory stock
LMS4209	K-12 (ATCC 23716) *ytfE*::Cm^r	25
S. aureus strains		
NCTC 8325	Parental strain	Laboratory stock
RN4220	Restriction-negative derivative of NCTC 8325; transformable by electroporation	Laboratory stock
LMSA0229	RN4220 *scdA*::Erm^r	This study
N. gonorrhoeae strains		
F62	Parental strain	Laboratory stock
JCGC704	*dnrN*::Kan^r	This study
JCGC212	*kat*::*ermC*	51
JCGC705	*kat*::*ermC dnrN*::Kan^r	This study
Plasmids		
pSP64D-E	Cloning vector carrying an erythromycin resistance cassette	8
pSPScdA	Upstream region and 5′ end of *scdA* cloned into pSP64D-E, next to the erythromycin resistance cassette	This study
pSUB11	Epitope tagging plasmid carrying three copies of the FLAG tag and a kanamycin resistance cassette	55
pGEM-T Easy	Cloning vector	Promega
pGEMDnrN	Sequences upstream and downstream of the *dnrN* gene (NGO0653) cloned into pGEM-T Easy	This study
pGEMDnrN-KO	Sequences upstream and downstream of the *dnrN* gene (NGO0653) flanking a kanamycin resistance cassette cloned into pGEM-T Easy	This study
pET28a(+)	T7-based expression vector that inserts a sequence encoding a His_6 tag at the N terminus	Novagen
pET-ScdA	*S. aureus scdA* gene cloned into pET28a(+)	This study
pGS57	Fumarase A-expressing plasmid	57
pUC18	Cloning vector	Laboratory stock
pScdA	pUC18 carrying the *scdA* gene of *S. aureus* and its promoter region	This study
pYtfE	pUC18 carrying the *ytfE* gene of *E. coli* and its promoter region	24
pRKISC	Plasmid for expression of the *E. coli isc* operon	51
pRKSUF	Plasmid for expression of the *E. coli suf* operon	52

cludes various iron-sulfur-containing enzymes and the transcription factors that regulate their synthesis, which is a reflection of the fact that iron-sulfur centers are damaged by both reactive oxygen and nitrogen species. In addition, the perturbation of iron homeostasis that occurs under stress conditions causes changes in the transcriptional regulation of a large number of genes involved in iron metabolism, many of which code for iron-containing proteins (28, 36).

Analysis of the data available for the gram-positive pathogen *Staphylococcus aureus* and for the gram-negative pathogens *Neisseria meningitidis and Neisseria gonorrhoeae*, organisms that have serious impacts on human health, revealed that exposure to nitric oxide and hydrogen peroxide causes the induction of genes encoding putative iron-containing proteins. Examples include the *S. aureus scdA*, whose expression was reported to be induced by both NO and hydrogen peroxide (4, 44), and the gonococcal *dnrN*, which is induced when the NsrR repressor protein is inactivated by NO (41). We therefore investigated whether either of these proteins is implicated in protection against nitric oxide or hydrogen peroxide, reactive nitrogen and oxygen species generated by the human body as part of its defenses against infection by pathogenic bacteria, and in iron metabolism. The results of in vivo and in vitro experiments revealed a role for these proteins in the repair of iron-sulfur centers of both transcription factors and housekeeping enzymes damaged by oxidative and nitrosative stress. Furthermore, the analysis of protein databases emphasizes their importance since related proteins were found in a wide range of prokaryotic and eukaryotic pathogens.

MATERIALS AND METHODS

Strains, plasmids, and primers. Bacterial strains and plasmids used in this work are listed in Table 1, and oligonucleotides are listed in Table S1 in the supplemental material. To disrupt the *scdA* gene (SAOUHSC_00229) of *S. aureus* NCTC 8325, an 820-bp fragment spanning the upstream region and 5′ end of the gene was amplified by PCR using the primers ScdAmutEco and ScdAmutBam, and the fragment was cloned into pSP64D-E (17). The resulting plasmid, pSPScdA, was electroporated into *S. aureus* RN4220, and transformants were selected on tryptic soy agar (TSA; Difco) plates containing erythromycin (10 μg/ml). The correct integration of pSPScdA into the chromosome of RN4220 in the strain obtained, LMSA0229 (*scdA*::Erm^r), was confirmed by single-colony PCR analysis.

The *dnrN* gene of *N. gonorrhoeae* (open reading frame NGO0653) was interrupted with a kanamycin resistance cassette using crossover PCR (31). Primers DnrNA plus DnrNB and DnrNC plus DnrND were used to generate DNA fragments upstream and downstream of the *dnrN* gene. The flanking fragments were cleaned and combined in a crossover PCR with primers DnrNA and DnrND, yielding a single fragment with an AgeI restriction site between the upstream and downstream sequences. The crossover PCR product was cloned into pGEM T-Easy (Promega, Madison, WI), yielding pGEMDnrN. A kanamycin resistance cassette was amplified from pSUB11 by PCR using primers KanAgeIFwd and KanAgeIRev, which introduced AgeI sites at each end of the resultant fragment, and was ligated into AgeI-digested pGEMDnrN, yielding pGEMDnrN-KO. The *dnrN*::*kan* fragment was generated by digestion of pGEMDnrN-KO with EcoRI and was transformed, as previously described (32), into piliated *N. gonorrhoeae* strain F62, yielding strain JCGC704.

Growth of *S. aureus* and sensitivity assays. *S. aureus* RN4220 and LMSA0229 strains were streaked onto TSA plates and incubated for 16 h at 37°C. Isolated colonies were cultivated aerobically in tryptic soy broth medium (Difco) for 16 h at 37°C and 150 rpm. These were used to inoculate, in duplicate, 20 ml of fresh tryptic soy broth, adjusting the starting optical density at 600 nm (OD_{600}) to 0.1. The cultures, grown aerobically at 37°C, were treated with 10 mM H_2O_2 or left untreated. After 4 h of growth, 5 μl of serial dilutions of the cultures was spread onto TSA plates and incubated overnight.

Growth of *N. gonorrhoeae* and sensitivity to hydrogen peroxide. *N. gonorrhoeae* was grown on gonococcal agar plates and in gonococcal broth (GCB; BD, Oxford, United Kingdom). Solid and liquid media were supplemented with 1% (vol/vol) Kellogg's supplement (26). For liquid cultures, 2 μl of a stock of *N. gonorrhoeae* was plated onto a gonococcal agar plate and incubated in a candle jar at 37°C for 24 h. Bacteria from this plate were swabbed onto a second plate and incubated in the same way for a further 16 h. The entire bacterial growth from this second plate was swabbed into 10 ml of GCB and incubated at 37°C in an orbital shaker at 100 rpm for 1 h. This 10-ml preculture was then transferred into 50 ml of GCB in a 100-ml conical flask and incubated in the same way. For growth in the presence of nitrite, 1 mM $NaNO_2$ was added after 1 h, and 4 mM $NaNO_2$ was added 1 h later.

A modified disk diffusion assay was used to compare areas of growth inhibition of various gonococcal strains (54). For growth experiments, different concentrations of H_2O_2 were added to 60 ml of oxygen-limited cultures of the gonococcal *kat* mutant and the *kat dnrN* double mutant in 100-ml conical flasks, and growth was monitored for the following 5 h. Greatest differences between the two strains were observed when the H_2O_2 concentration added was 0.5 mM.

Complementation assays in *E. coli*. A DNA fragment of 955 bp comprising the promoter and coding regions of *scdA* was amplified by PCR from *S. aureus* NCTC 8325 genomic DNA, using the primers ScdAHindIII and ScdAEcoRI, and cloned into pUC18 digested with HindIII and EcoRI, generating the plasmid pScdA. The *E. coli ytfE* mutant strain LMS4209 was transformed with the plasmids pYtfE, pScdA, pRKISC, and pRKSUF that express, respectively, the *E. coli ytfE* gene, *S. aureus scdA* gene, *E. coli isc* operon, and the *suf* operon from their own promoters. *E. coli* strains were grown in LB medium under anaerobic conditions (i.e., closed flasks completely filled), from a starting OD_{600} of 0.1. When cultures reached an OD_{600} of 0.3, they were treated with 4 mM hydrogen peroxide (Sigma) or left untreated, and the growth was followed for ~3 h.

Production of the *S. aureus* recombinant ScdA protein. The coding region of the *scdA* gene was amplified by PCR from genomic DNA of *S. aureus* NCTC 8325 using the primers ScdANheI and ScdAEcoRI and cloned into pET-28a (Novagen) that allows insertion of a nucleotide sequence that encodes a His_6 tail at the N terminus. The resulting plasmid, pET-ScdA, was sequenced to ensure the integrity of the cloned sequence. The recombinant protein was overproduced in cells of *E. coli* BL21 Gold(DE3) (Stratagene) grown aerobically in M9 minimal medium, which was supplemented with 10 mM glucose, 100 μM $Fe(NH_4)_2(SO_4)_2$, and 30 μg/ml kanamycin; cells were cultured at 37°C and 150 rpm. At an OD_{600} of 0.3, the cultures were induced with 400 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG). After the temperature was lowered to 30°C, cultures were grown for 6 h at 130 rpm and harvested by centrifugation. Cells were resuspended in ice-cold buffer A (20 mM Tris-HCl, pH 7.6), disrupted in a French press, and ultracentrifuged at 100,000 × *g* for 2 h at 4°C. The soluble extract was loaded onto an immobilized metal affinity chromatography Sepharose Fast Flow column (GE Healthcare), and ScdA was eluted at 300 mM imidazole and immediately dialyzed against buffer A. The protein was found to be pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and this sample was used for further characterization.

Protein concentration was determined by a bicinchoninic acid protein assay (Pierce) (49), and the iron content was determined by the TPTZ (2,4,6-tripyridyl-1,2,3-triazine) method (11). Molecular mass determination was performed in a Superdex 200 (10/300) GL column (GE Healthcare) using standard proteins. The electron paramagnetic resonance (EPR) spectrum was obtained in a Bruker EMX spectrometer equipped with an Oxford Instruments continuous-flow helium cryostat and was recorded at a 9.39-MHz microwave frequency with 2.4 mW of microwave power at 10 K.

Repair of the damaged [4Fe-4S] cluster of *E. coli* fumarase A. Cells of the *E. coli ytfE* mutant strain transformed with pGS57 were grown aerobically with 1 mM IPTG to an OD_{600} of ~0.5, collected by centrifugation, resuspended (1/100) in fumarase assay buffer (35), and lysed by four freeze-thaw cycles. Two minutes before the stresses were imposed, 100 μg/ml tetracycline was added to the cell extracts to inhibit de novo protein synthesis. After 1 min of incubation with 4 mM H_2O_2 or 5 min with 150 μM NO, 400 U/ml of catalase or 40 μM hemoglobin (Sigma) was added, respectively, and the fumarase activity was determined at fixed time points. Purified ScdA protein was added at a final concentration of 20 μM immediately after the stresses were removed.

Fumarase activity was determined spectrophotometrically by following the disappearance of fumarate as described by Massey (35). The cell samples were quickly thawed at room temperature, cleared by the addition of 0.5% (wt/vol) sodium deoxycholate, and then diluted in 50 mM sodium phosphate buffer, pH 7.3. The reactions were started by the addition of 10 mM fumarate and followed (at 295 nm, $\varepsilon = 0.07\ mM^{-1}\ cm^{-1}$). Enzyme activities were determined at 25°C and are defined as units (μmol of fumarate consumed per min) per mg of total protein. The enzyme activities were determined in duplicate from two independent cultures and are presented as averaged values, with error bars representing one standard deviation.

Determination of the aconitase activity in *S. aureus*. Aconitase activity was determined in cell lysates of *S. aureus* RN4220 and the *scdA* mutant that had been grown aerobically in LB medium at 37°C to an OD_{600} of 0.5. The cells were collected by centrifugation, resuspended (1/200) in assay buffer (50 mM Tris-HCl, pH 7.7, 0.6 mM $MnCl_2$), and lysed by a 10-min incubation at 37°C with 75 μg/ml lysostaphin. Cell lysates were exposed to 100 μM NO or 3 mM H_2O_2, and at specific times aliquots were frozen in liquid nitrogen and later assayed. To monitor the repair of the damaged enzyme, the lysates were treated with tetracycline (100 μg/ml) prior to exposure to H_2O_2 for 5 min or NO for 15 min. Upon addition of catalase (400 U/ml) or hemoglobin (40 μM), the aliquots were collected and frozen. Aconitase activity was determined by following the formation of NADPH through the indirect method described by Gardner (16). Samples were quickly thawed at room temperature, cleared by the addition of 0.5% (wt/vol) sodium deoxycholate, and immediately inserted into suba-sealed cuvettes with deaerated assay buffer that contained 0.2 mM $NADP^+$ and 1 U of isocitrate dehydrogenase (Sigma). The reaction was initiated with 50 mM sodium citrate. Aconitase activities determined at 25°C in duplicate from two independent cultures are defined as units (μmol of NADPH formed per min) per mg of total protein and are presented as averaged values, with error bars representing one standard deviation.

Quantitative real-time PCR analysis of gene expression. Relative gene expression was measured using quantitative reverse transcription-PCR (qRT-PCR) as described previously (41). RNA was stabilized by mixing 500 μl of bacterial culture with 900 μl of RNAlater solution (Ambion). After a 5-min incubation at room temperature, the bacteria were harvested by centrifugation at 3,000 × *g* for 10 min. RNA was isolated from the pellet using an RNeasy mini kit (Qiagen) using the manufacturer's protocol. Genomic DNA was removed from the purified RNA using Turbo DNase (Ambion). The RNA was reverse transcribed to cDNA using a Superscript first-strand synthesis kit (Invitrogen). For each sample, a control to check for DNA contamination in the RNA preparation was included from which reverse transcriptase was omitted. Transcript levels were measured by quantitative real-time PCR using SensiMix with Sybr green detection (Quantace) and an ABI 7000 sequence analyzer (Applied Biosystems). Primers designed using PrimerExpress (Applied Biosystems) are described in Table S1 in the supplemental material. Transcript levels were quantified using the $\Delta\Delta C_T$ (where C_T is threshold cycle) method (33) relative to expression of the *polA* gene. Expression levels were normalized for each strain prior to shock with nitrite. For each experiment, quantitative real-time PCR was used to determine transcript levels on three independent cDNA samples derived from three independent cultures.

Determination of rates of NO reduction by washed bacterial suspensions. *N. gonorrhoeae* strain F62 and its *dnrN* mutant were grown as described above in oxygen-limited cultures supplemented with nitrite and harvested by centrifugation, and the rates of NO reduction were assayed using a Hansatech Instruments oxygen electrode adapted for increased sensitivity to NO (53). All solutions used for these assays were purged of oxygen for at least 10 min using oxygen-free nitrogen gas. The concentration of NO at the start of the assay was 200 μM, and the bacterial density assayed was in the range of 1 to 2 mg of dry mass ml^{-1}.

RESULTS AND DISCUSSION

Effect of a mutation in *S. aureus scdA* and *N. gonorrhoeae dnrN* on recovery from oxidative and nitrosative stress. To assess the function of ScdA in *S. aureus*, the effects of NO and H_2O_2 on growth of an *scdA* mutant and its parent were compared. The *scdA* mutant strain showed no morphological defects, contrary to what had been previously described (3). Although no differences could be detected between the wild-type and mutant strains in response to exposure to NO (data not

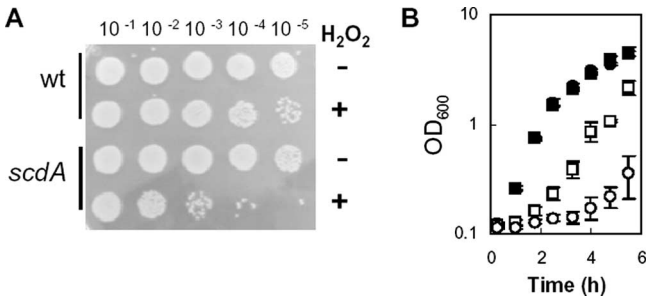

FIG. 1. The sensitivity of *S. aureus* to hydrogen peroxide increases in the absence of *scdA*. (A) Serial dilutions of cultures of *S. aureus* RN4220 (wt) and the *scdA* mutant strain after 4 h of growth in the presence (+) or absence (−) of 10 mM H_2O_2. A representative plate of independent experiments performed in duplicate is shown. (B) Growth of *S. aureus* RN4220 (squares) and the *scdA* mutant (circles) monitored by the OD_{600} nm in cultures untreated (filled symbols) or treated with 10 mM H_2O_2 (open symbols). Mean values of two independent cultures are given, with error bars showing the standard deviations.

shown), the *scdA* mutant was more sensitive to oxidative conditions than its parent (Fig. 1). Hence, ScdA constitutes an efficient protection system against hydrogen peroxide.

In *N. gonorrhoeae*, binding sites for the NO-sensitive transcription factor NsrR, a member of the Rrf2 family of transcription factors, were identified at the promoters of *aniA*, which controls the expression of the gene encoding a copper-containing nitrite reductase similar to NirK in other bacteria; *norB*, encoding the single subunit nitric oxide reductase; and a gene of unknown function, *dnrN*. All of these genes are now known to be induced upon exposure to nitric oxide (41, 45). The gonococcal DnrN protein is 16% identical and 31% similar in amino acid sequence to *S. aureus* ScdA, suggesting that it might be a functional homologue of ScdA. A *dnrN* deletion mutant was constructed, and the effects of the mutation on recovery from exposure to NO were assessed. As the gonococcal *dnrN* gene is monocistronic, the possibility of secondary effects of the mutation on downstream genes was discounted. Since gonococci generate NO as the product of nitrite reduction during oxygen-limited growth, it was predicted that the *dnrN* mutant might be more sensitive to sudden exposure to nitrite, which will be converted rapidly to NO, than its $dnrN^+$ parent. Sudden addition of nitrite to a culture in which AniA has accumulated but NorB synthesis has not been induced will lead to the sudden generation of NO, which would cause damage from which only the parent strain can recover. In contrast, if *aniA* and *norB* transcription are gradually induced sequentially because nitrite is available during adaptation to oxygen-limited growth, both the mutant and the parent are able to adapt.

To test these predictions, the mutant and parent strains were first grown in oxygen-limited medium supplemented with 5 mM nitrite. FNR, the regulator of fumarate and nitrate reduction during anaerobic growth, is essential in gonococci for expression of several genes including the nitrite reductase *aniA*. In the experiment, as the cultures became oxygen-limited, FNR gradually became activated, inducing the transcription of *aniA*. The consequent production of NO, generated during nitrite reduction, induced synthesis of the gonococcal nitric oxide reductase NorB, which scavenges the NO present in the cell. We propose that, under these conditions, the concentration of NO available is low; therefore, the mutant grew only slightly more slowly than the parent, and growth of both strains stopped at similar cell densities (Fig. 2A).

Parallel cultures were also grown in the absence of nitrite. Under this condition, expression of the nitrite reductase *aniA* would still occur (32, 56), but as NO would not be formed, *norB* would remain repressed by NsrR. When the cultures became oxygen limited, nitrite was added, resulting in a sudden pulse of NO generation. As predicted, after an initial inhibition of growth the parent strain, F62, recovered, but growth inhibition of the *dnrN* mutant persisted (Fig. 2B). It was concluded that the gonococcal *dnrN* mutant is more sensitive than its parent to damage induced by a sudden exposure to nitric oxide generated from nitrite.

An NO-sensitive electrode was used to eliminate an alternative possibility, i.e., that the *dnrN* mutant is defective in its ability to reduce NO compared with the parent strain (50). The rates of NO reduction by bacteria harvested from these cultures were measured. The average values for the two strains were indistinguishable, 162 (±18) nmol of NO reduced $min^{-1} \cdot$ mg of bacterial dry $mass^{-1}$ for the mutant compared with 164 (±46) nmol of NO reduced $min^{-1} \cdot$ mg of bacterial dry $mass^{-1}$ for the parental strain. Furthermore, these rates of NO reduction were sufficiently high to exclude the possibility that NO accumulates to a higher concentration in cultures of the mutant, causing more severe or even different types of damage (50).

Pathogenic neisseria synthesize an extremely active catalase that masks any protective functions of other proteins that protect the bacteria from exposure to hydrogen peroxide (22). To reveal whether DnrN plays any role in protection against oxidative stress, the *dnrN* deletion mutation was transferred into *N. gonorrhoeae* strain JCGC212, from which the *kat* gene has been deleted. The effects of exposure to hydrogen peroxide on growth of both the *dnrN kat* double mutant and its isogenic $dnrN^+$ strain in liquid medium were then compared. Despite the absence of catalase activity, at very low concentrations of H_2O_2 (<0.5 mM), growth of neither the mutant nor the parental strain was significantly inhibited. Conversely, growth of both strains was completely inhibited at a high concentration of H_2O_2. However, the *dnrN* mutant was more sensitive than its parent at an intermediate concentration of H_2O_2 (Fig. 2C). Disk diffusion assays confirmed that the *kat dnrN* double mutant was also more sensitive than the *kat* single mutant to growth in the presence of hydrogen peroxide on solid medium (Fig. 2D). These results implicated DnrN in protection against not only nitrosative stress but also oxidative stress.

Increased sensitivity of the gonococcal *dnrN* mutant to damage to iron-sulfur centers of the transcription factors FNR and NsrR. The results presented above established that strains mutated in the gonococcal *dnrN* and in *S. aureus scdA* have increased susceptibility to exogenous hydrogen peroxide. This phenotype is frequently correlated with elevated levels of intracellular free iron to which the degradation of iron-sulfur centers contributes (27). In addition, one possible explanation for the sensitivity of the gonococcal *dnrN* mutant to nitrosative stress is that sudden exposure to NO damaged the iron-sulfur centers of FNR, NsrR, and also many other iron-sulfur pro-

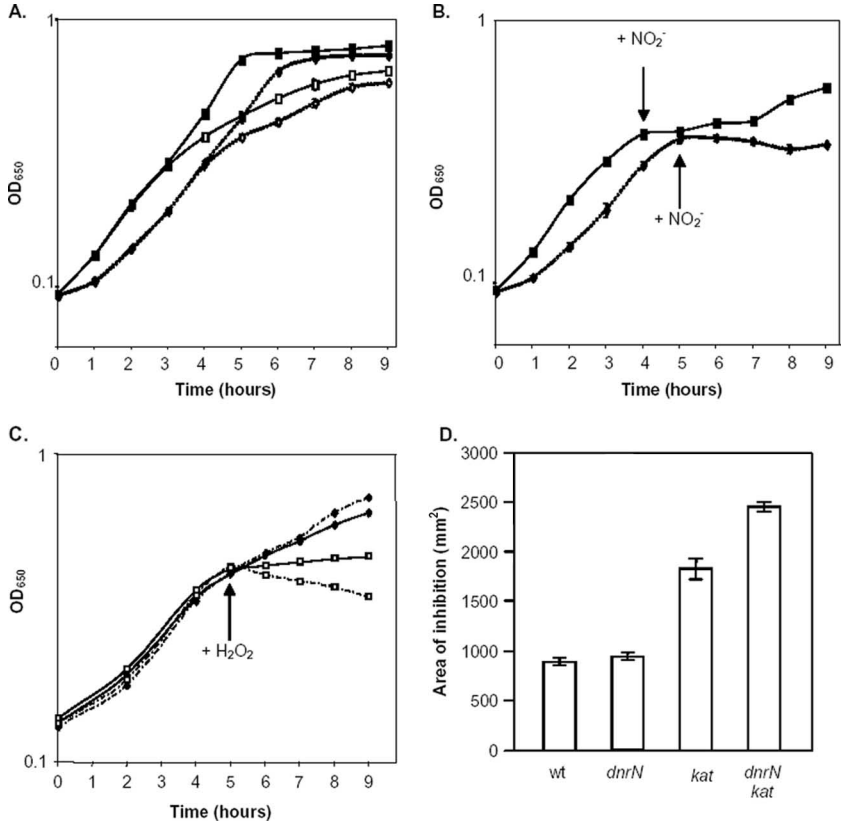

FIG. 2. Effect of a *dnrN* mutation on the recovery of *N. gonorrhoeae* from damage induced by sudden exposure to nitric oxide or hydrogen peroxide. (A and B) Deletion of *dnrN* results in a growth phenotype in *N. gonorrhoeae*. The OD_{650}s of oxygen-limited cultures of *N. gonorrhoeae* strains F62 (*dnrN*$^+$; solid lines) and JCGC704 (*dnrN*; dotted lines) were measured at hourly intervals. (A) Growth in the presence (filled symbols) or absence (open symbols) of 5 mM nitrite. (B) Growth in the absence of nitrite until an OD_{650} of around 0.4 (about 0.16 mg of dry mass ml^{-1}) was reached, followed by shock with 0.5 mM $NaNO_2$ (arrows). Error bars show standard deviations of duplicate cultures. (C and D) Effect of a hydrogen peroxide on the growth of *kat* and *dnrN kat* mutants. Oxygen-limited cultures of *N. gonorrhoeae* JCGC212 (*kat*; solid lines) and JCGC807 (*dnrN kat*; dotted lines) were grown in the absence of nitrite (C). Half of the cultures were shocked with 0.5 mM hydrogen peroxide at an OD_{650} of around 0.4 (shown by arrow; open squares) while the remaining cultures were not treated (filled diamonds). The growth experiment was repeated twice. *N. gonorrhoeae* strains F62 (wild-type), JCGC704 (*dnrN*), JCGC212 (*kat*), and JCGC807 (*dnrN kat*) were first grown on GC agar plates for 1 day at 37°C (D). A lawn of each strain was spread onto a fresh GC agar plate supplemented with 1 mM sodium nitrite. A 12-mm filter paper disc was seeded in the center of the plate with 10 μl of 30% hydrogen peroxide, and plates were incubated for 4 days at 37°C in an anaerobic jar. The area of growth inhibition was calculated. Error bars are standard deviations of triplicate samples.

teins. As there is currently no system for overexpressing proteins in the gonococcus, the strategy devised to demonstrate the role for DnrN in repair of nitrosative damage was to monitor by quantitative real-time PCR the accumulation of mRNA synthesized under the control of the two transcription factors, FNR and NsrR, in which iron-sulfur centers are critical for function. First, we exploited the NO-induced damage to the oxygen-sensing $[4Fe\text{-}4S]^{+2/+1}$ iron-sulfur center of FNR that results in loss of DNA binding and transcription activation and consequent loss of *aniA* expression. The qRT-PCR experiments showed that the loss of *aniA* expression immediately after exposure to NO was followed by restoration of the accumulation of *aniA* mRNA in the parental strain but not in the mutant (Fig. 3A). This result confirmed that the damage was repaired more rapidly in a parental strain, F62, than in a *dnrN* mutant.

The NsrR protein, which is also predicted to contain an Fe-S center (2), represses the expression of the *norB* gene, but repression is lifted on exposure to low concentrations of NO (20, 41). If the interpretation of the effects of a *dnrN* mutation on *aniA* expression is correct, it can be predicted that exposure to NO would result in a rapid increase in *norB* expression. Repression would be restored rapidly in the parental *dnrN*$^+$ strain but not in a *dnrN* mutant. This prediction was confirmed (Fig. 3B). Furthermore, *dnrN* mRNA also accumulated rapidly in the parental strain following NO exposure, but the level of this transcript also decreased rapidly as NsrR repression was restored (Fig. 3C).

In the absence of *S. aureus scdA*, the activity of the iron-sulfur enzyme aconitase is decreased. *S. aureus* synthesizes a single aconitase, a dehydratase of the tricarboxylic acid cycle, that contains a $[4Fe\text{-}4S]^{+2/+1}$ cluster that is susceptible to damage by NO and oxidants such as hydrogen peroxide. In the absence of *scdA*, the activity of aconitase was found to be 33% lower than in the *S. aureus* parent strain. Furthermore, when cell lysates of *S. aureus* were exposed to NO or to hydrogen peroxide, a faster decrease of the aconitase activity was observed in the *scdA* mutant than in its parent (Fig. 4A and C).

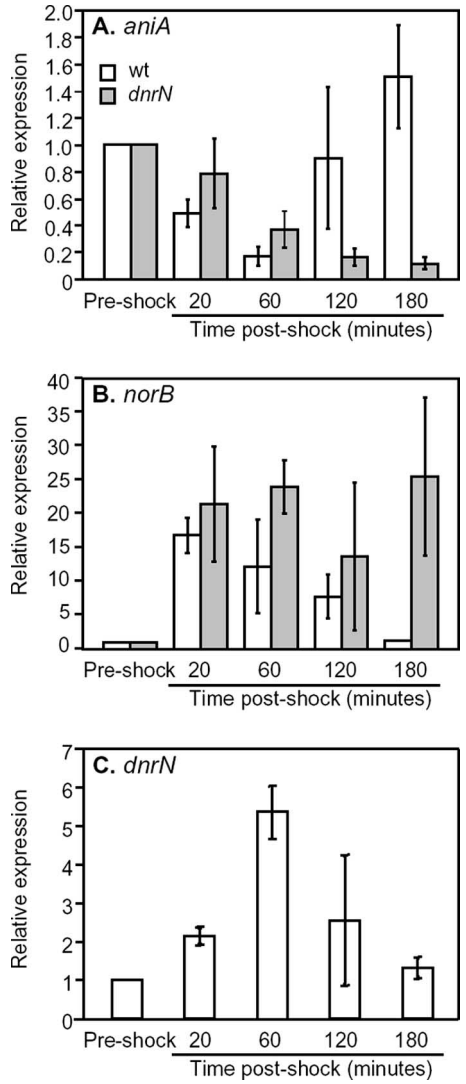

FIG. 3. qRT-PCR analysis of gene expression before and after shock with nitrite. *N. gonorrhoeae* strains F62 (parent) and JCGC704 (*dnrN*) were grown under oxygen-limited conditions in the absence of nitrite to an OD_{650} of ~0.4 and then shocked with 0.5 mM $NaNO_2$. RNA was isolated preshock and 20, 60, 120, and 180 min after the shock, and quantitative PCR was used to quantify *aniA* (A), *norB* (B), and *dnrN* (C) transcripts. Quantities are normalized against the preshock transcript level for each strain.

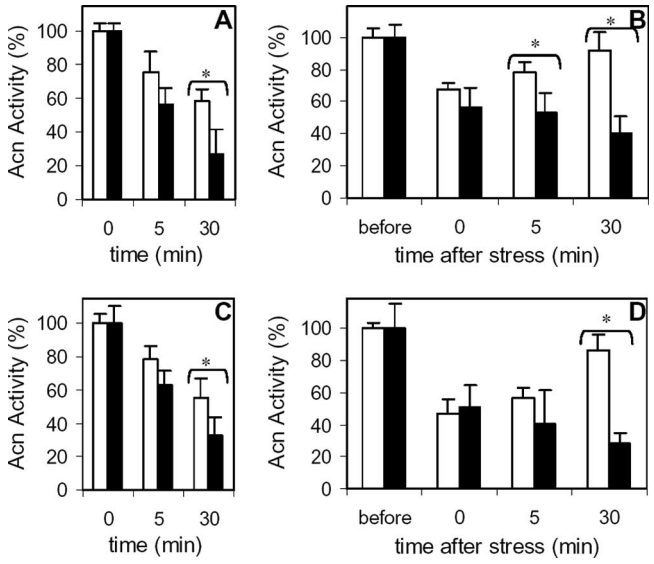

FIG. 4. Nitric oxide and hydrogen peroxide-induced damage to aconitase is more pronounced, and the repair of the damage is severely impaired in the absence of *scdA*. Cell lysates of the *S. aureus* RN4220 parent strain (open bars) and the *scdA* mutant (filled bars) were subjected to 3 mM H_2O_2 (A and B) or 100 μM NO (C and D). For the time course of damage (A and C) the aconitase activity was monitored for 30 min. To follow the repair of aconitase, after 2 min with H_2O_2 (B) or 15 min with NO (D), catalase and hemoglobin were added to interrupt the exposures (time zero), and the activity was then monitored. The values are averages of duplicate determinations from two (B and D) or four (A and C) independent experiments, with error bars representing one standard deviation unit. The asterisk represents statistical significance ($P < 0.05$) using a Student's *t* test. The values are normalized for the initial activity of each strain (wild type, 17.1 mU/mg protein; *scdA*, 11.5 mU/mg protein).

We also tested the influence of ScdA in the recovery of aconitase activity upon damage caused by oxidative or nitrosative stress. The aconitase activities of cell lysates prepared from each culture during subsequent incubation in the absence of NO or H_2O_2 were then assayed. Tetracycline was added to cultures of each strain to inhibit de novo protein synthesis, and, after a brief exposure to NO or H_2O_2, hemoglobin or catalase was added to scavenge excess NO or H_2O_2. Aconitase activity was restored rapidly only in the parental strain (Fig. 4B and D).

Major contribution of *S. aureus* di-iron ScdA to repair of stress-induced damage to the iron-sulfur center of fumarase. The phenotype of the *S. aureus scdA* mutant resembles that recently described for an *E. coli ytfE* mutant. In both cases, the activities of iron-sulfur-containing enzymes are lower in the mutant (23). To determine whether ScdA and YtfE also showed similar biochemical properties, the recombinant *S. aureus* ScdA was produced in *E. coli* and characterized. The purified ScdA protein was isolated as a dimer with a molecular mass of 57 kDa and was found to contain two iron atoms per monomer. The visible spectrum exhibited a broad band at 350 nm, characteristic of iron-containing proteins (data not shown). *S. aureus* ScdA exhibited an EPR spectrum with *g* values of 1.96, 1.92, and 1.86 (Fig. 5), which are within the range of values usually observed for proteins containing di-iron centers, including the *E. coli* YtfE (23, 30).

The similarity between the *E. coli* YtfE and *S. aureus* ScdA

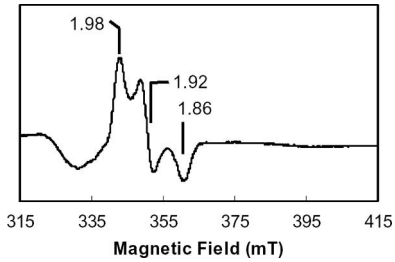

FIG. 5. ScdA protein of *S. aureus* has a di-iron center. EPR spectrum of the as-prepared ScdA protein, recorded at 10 K at a 9.4-MHz microwave frequency with 2.4 mW of microwave power.

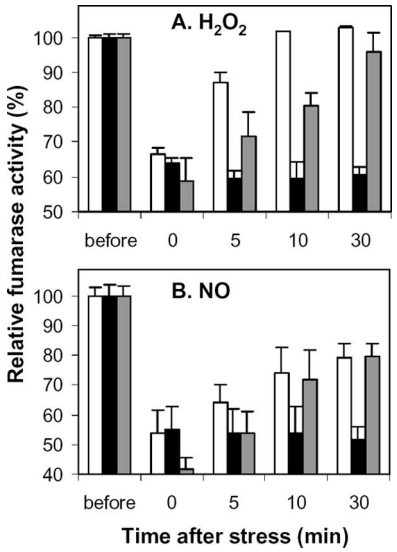

FIG. 6. The ScdA protein of *S. aureus* repairs the [4Fe-4S] cluster of fumarase A after damage by nitric oxide and hydrogen peroxide. Fumarase activity was monitored in lysates of *E. coli* K-12 cells (open bars) and *E. coli ytfE* mutant cells (black bars) expressing fumarase A after treatment with tetracycline and treatment with 4 mM H_2O_2 for 1 min (A) or 150 μM NO for 10 min (B). Immediately after terminating the stresses by the addition of catalase or hemoglobin, purified ScdA protein was added to *ytfE* mutant cell lysates (gray bars), and the activity was measured (time zero) and monitored for 30 min. The values are normalized for the initial activity ("before") of each strain (wild type, 3.7 U/mg protein; *ytfE*, 2.9 U/mg protein) and are mean values of two experiments analyzed in duplicate. Error bars represent one standard deviation unit.

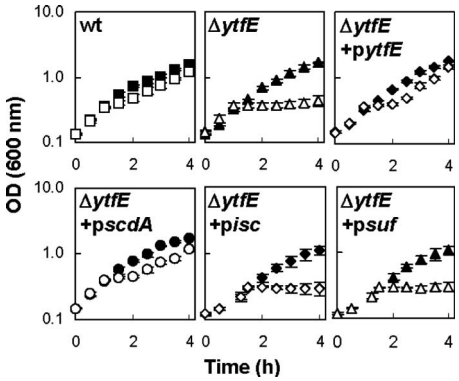

FIG. 7. *S. aureus scdA*, but not the *suf* or *isc* operons of *E. coli*, complement the sensitivity to hydrogen peroxide of the *E. coli ytfE* mutant. The *E. coli* K-12 parent strain (wt), *ytfE* mutant strain (*ytfE*), *ytfE* strain expressing *E. coli ytfE* in *trans* (*ytfE*/p*ytfE*), *ytfE* strain expressing *S. aureus scdA* in *trans* (*ytfE*/p*scdA*), *ytfE* strain expressing the *E. coli isc* operon in *trans* (*ytfE*/p*isc*), and *ytfE* strain expressing the *E. coli suf* operon in *trans* (*ytfE*/p*suf*) were grown in LB medium under anaerobic conditions. Cultures were left untreated (filled symbols) or treated with 4 mM H_2O_2 at an OD_{600} of ~0.3 (open symbols). Mean values of two independent experiments with error bars representing the standard deviations are shown.

proteins led us to investigate whether the recombinant ScdA could support the in vitro repair of a damaged [4Fe-4S] cluster, as shown for *E. coli* YtfE (24). Indeed, addition of purified ScdA protein to cell lysates of *E. coli* Δ*ytfE* expressing fumarase A and exposed to hydrogen peroxide (Fig. 6A) or nitric oxide (Fig. 6B) demonstrated that ScdA promotes restoration of the fumarase activity to the levels observed before damage. These results show that *S. aureus* ScdA is essential for the repair of an $[4Fe\text{-}4S]^{+2/+1}$ protein whose cluster is damaged by oxidative or nitrosative compounds.

***S. aureus scdA*, but not the *suf* or *isc* operons, complements the hydrogen peroxide sensitivity of the *E. coli ytfE* mutant.** Next, we addressed the question of whether *S. aureus scdA* could replace the function of *ytfE*. To this end, the *E. coli ytfE* mutant strain was transformed with plasmids encoding either the *E. coli ytfE* or the *S. aureus scdA* genes, and sensitivity of the strains was measured under oxidative stress conditions generated by hydrogen peroxide. The *ytfE* mutant was more sensitive to hydrogen peroxide than the parent, and hypersensitivity was suppressed by expression in *trans* of either the *E. coli ytfE* or the *S. aureus scdA* gene (Fig. 7).

The *E. coli isc* and *suf* operons are proposed to encode proteins that may also be involved in the repair of Fe-S clusters. However, the resistance of the *ytfE* mutant to hydrogen peroxide was not restored by either the plasmid pRKSUF or pRKISC (Fig. 7), containing the complete *sufABCDSE* or *iscRSUAhscBAfdx* operon of *E. coli*, respectively (51, 52). Hence, the ISC and SUF systems cannot replace YtfE, even though SUF is reported to operate under such stress conditions as oxidative stress and iron starvation (38, 40). Note, however, that the plasmid containing the complete set of *suf* genes could complement most defects of the Δ*iscRSUA hscBAfdx* strain (52). We conclude that *S. aureus* ScdA and *E. coli* YtfE have similar biochemical roles.

Phylogenetic analysis of ScdA and DnrN homologues. The amino acid sequences of *S. aureus* ScdA and gonococcal DnrN share 25 and 31% identity and 46 and 41% similarity to *E. coli* YtfE, respectively. Moreover, a comprehensive search of the amino acid sequence database revealed that DnrN, ScdA, and *E. coli* YtfE are members of a large family of proteins that occur widely in the bacterial phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Acidobacteria*. In particular, homologues of these proteins are encoded in the genomes of a significant number of human pathogens, such as *Bacillus anthracis*, *Haemophilus influenzae*, and species of the genera *Salmonella*, *Shewanella*, *Yersinia*, and *Clostridium*. Interestingly, two orthologous sequences were found in the eukaryotic organism *Trichomonas vaginalis*, which is also a human pathogen.

Since a recent study in the pathogenic yeast *Cryptococcus neoformans* suggests that this eukaryote contains a homologue of the *E. coli* YtfE (5), this protein (CNA2870) and other putative fungal homologues were included in the analysis, in spite of the low sequence similarity of CNA2870 to the ScdA, DnrN, and YtfE proteins (7 to 8% identity and 16 to 17% similarity). Using all the above-mentioned amino acid sequences, a dendrogram was constructed (Fig. 8) that shows two main groups, one that includes the ScdA/DnrN/YtfE-like proteins and the other that includes the CNA2870-like proteins, in agreement with the low identity (3 to 11%) and similarity (9 to 22%) values between the sequences from both groups. The group of the ScdA/DnrN/YtfE-like proteins is apparently divided into two other groups, one comprising the majority of the

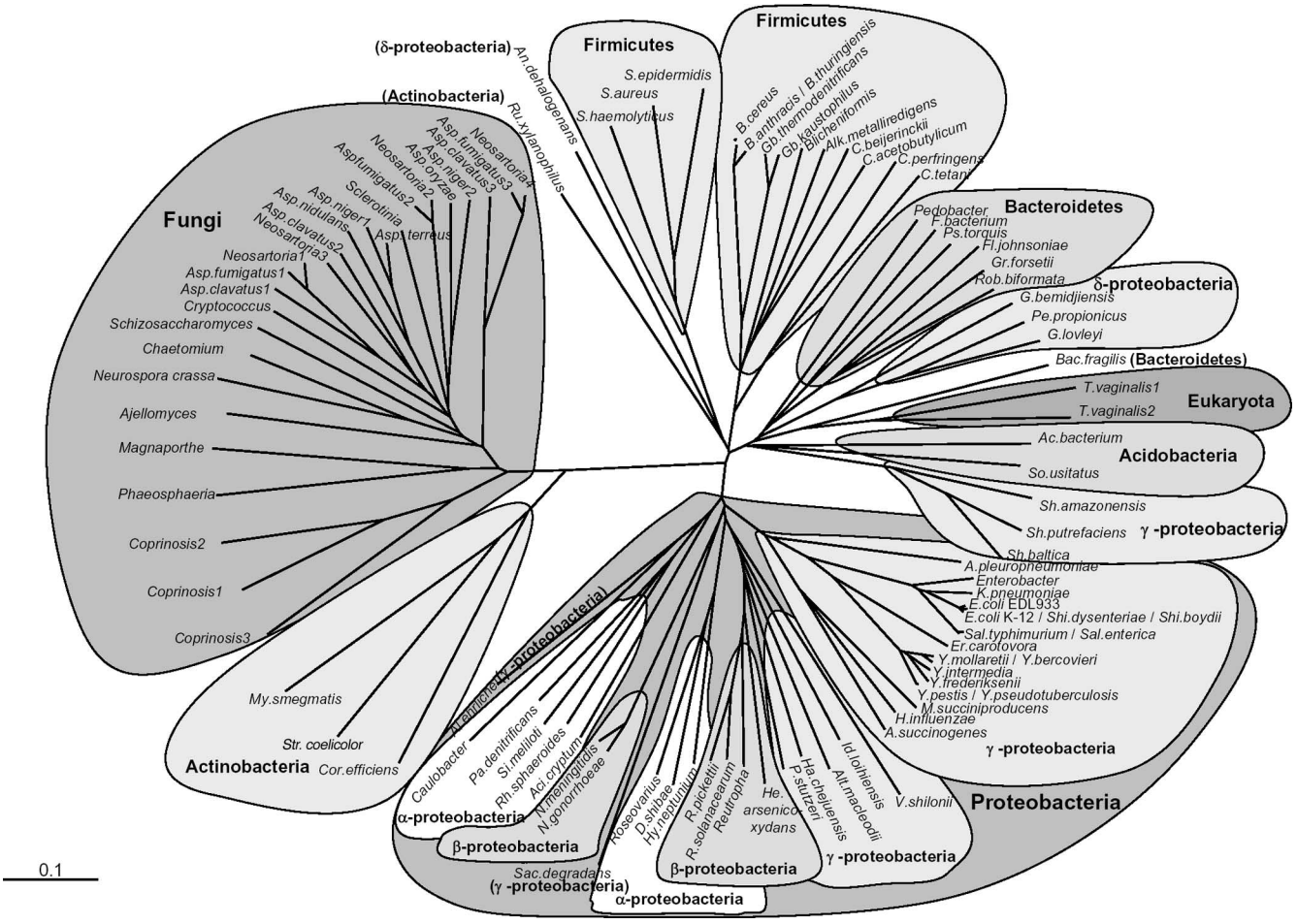

FIG. 8. Unrooted dendrogram of ScdA/DnrN family of proteins. The dendrogram was generated with Clustal X and manipulated in TreeView. A total of 102 sequences from *S. aureus* ScdA and *N. gonorrhoeae* DnrN homologues were aligned, and the dendrogram was bootstrapped by exclusion gap positions and correcting for multiple substitutions. Shaded boxes distinguish the different taxonomic groups. Abbreviations for the organisms are defined in the legend of Fig. S1 in the supplemental material.

proteobacteria and another containing the sequences of several taxa. The ScdA protein of *S. aureus* and the DnrN protein of *N. gonorrhoeae* are clustered separately due to the low amino acid sequence identity between the two proteins (16%).

The alignment of the amino acid sequences of the proteins (see Fig. S1 in the supplemental material) that produced the dendrogram in Fig. 8 revealed conservation of some regions (particularly within the ScdA/DnrN/YtfE-like sequences) and a high degree of conservation of the residues His^{84}, His^{105}, His^{129}, Glu^{133}, His^{160}, and His^{204} (numbering refers to residues in *E. coli* YtfE). Exceptions are observed for three yeast-like sequences in which Glu^{133} was replaced by an Asp. Based upon studies with *E. coli* YtfE, these residues are proposed to constitute the ligand sphere for the di-iron center (our unpublished results). In particular, they are located in conserved α-helix regions of a predicted secondary structure (see Fig. S1 in the supplemental material), corroborating the importance for the function of the four-helix-bundle protein fold that is predicted for ScdA/DnrN/YtfE and characterizes many other di-iron proteins.

RIC, a new family of proteins involved in the repair of iron centers. The work presented above has revealed the presence in a wide range of human, animal, and plant pathogens of a family of di-iron proteins that have similar functions. Based upon in vivo and in vitro evidence, we have shown that these proteins are present in both gram-positive and gram-negative bacteria and that the two main branches of this protein family can repair Fe-S clusters damaged by exposure to NO and H_2O_2. Our work corroborates and significantly extends the proposal of Rodionov et al. (45), based on the bioinformatics analysis of complete genome sequences, that DnrN in pathogenic *Neisseria* is involved in the response to nitrosative stress. Future research must focus on the exact chemical reactions catalyzed by this protein family during the repair process, for example, removal of the nitrosated iron atoms or reinsertion of iron once the primary damage has been removed by other proteins. As it is not known whether the substrates on which these proteins work are limited to those with iron-sulfur centers, we propose the name RIC, for *r*epair of *i*ron *c*enters, for this new and widely distributed protein family.

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