Oxidative-stress-inducible qorA encodes an NADPH-dependent quinone oxidoreductase catalysing a one-electron reduction in *Staphylococcus aureus*

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This work characterized the putative quinone oxidoreductase gene (qorA) from *Staphylococcus aureus*. The deduced amino acid sequence indicated that the 333 aa protein contains an NAD(P)H-binding motif. A Northern blot analysis revealed that 2·6 kb and 1·4 kb signals were detected by using a qorA probe. Both the signals were enhanced under the presence of a redox-cycling agent, 9,10-phenanthrenequinone (PQ). It was also revealed that the expression of three genes, SA1988, SA1989 (qorA) and SA1990, was enhanced at the transcriptional level by PQ exposure. The results suggested that the 2·6 kb signal detected by the qorA probe was in two co-transcripts, i.e. SA1990–qorA and qorA–SA1988 were transcribed. Besides, primer extension analyses confirmed the enhancement of qorA and SA1990 transcripts. The GST (glutathione S-transferase)-tagged QorA protein was expressed in *Escherichia coli* and purified using a glutathione affinity column. In purification steps, a 36 kDa band co-purified with the GST–QorA, and it was detected even in the thrombin-cleaved fraction. N-terminal amino acid sequences for the 36 kDa protein revealed that it was an intact QorA. They showed that QorA formed a multimer under physiological conditions. The purified recombinant GST–QorA catalysed NADPH consumption in the presence of PQ as a substrate, but not NADH. To characterize the catalytic activity of QorA, superoxide anion that was generated through one-electron reduction of PQ and hydroquinone that was produced by two-electron reduction of PQ were measured. During reduction of PQ by GST–QorA, superoxide anion was generated, whereas a small amount of 9,10-dihydroxyphenanthrene (hydroquinone of PQ) was produced. These results suggest that the activity of QorA is similar to 6-Crystallin, catalysing an NADPH-dependent one-electron reduction of quinone.

**INTRODUCTION**

*Staphylococcus aureus* is a major human Gram-positive pathogen and it can grow under aerobic or microaerobic conditions. The natural habitats of this bacterium are the mucus membrane and human skin. Once infected with *S. aureus*, it is difficult to eliminate this pathogen, especially in the case of multiple drug-resistant strains (MRSA). *S. aureus* causes various kinds of infectious disease, ranging from skin abscesses to life-threatening symptoms through its infections. The skin and mucous membranes act as a primary line of defence against infection by *S. aureus*. However, when this pathogen enters the underlying tissues, the innate immune response components including macrophages play a primary defence role. Macrophages and other lymphocytes use toxic reactive oxygen species (ROS) such as the superoxide anion, hydrogen peroxide and hydroxyl radicals to support killing phagocytosed bacteria (Clements & Foster, 1999).

The same kinds of ROS are also produced under aerobic conditions and can damage essential biomolecules such as DNA, protein and lipids. To confront these ROS, *S. aureus* produces antioxidant enzymes including superoxide dismutase (SOD) (Clements et al., 1999; Valderas & Hart, 2001) and catalase (Sanz et al., 2000). SOD is a major important enzyme in scavenging superoxide anions, which are reduced to hydrogen peroxide. Subsequently, hydrogen peroxide is broken down to water by catalase. The existence of the other oxidative stress responsive proteins, e.g.,

Abbreviations: DAP, 9,10-diacetoxyphenanthrene; GST, glutathione S-transferase; PQ, 9,10-phenanthrenequinone; Qor, quinone oxidoreductase; ROS, reactive oxygen species; SD, Shine–Dalgarno; SOD, superoxide dismutase.

Supplementary data showing that staphylococcal QorA does not predominantly catalyse the two-electron reduction of PQ is available at http://mic.sgmjournals.org
alkyl hydroperoxide reductase and metallo-regulated gene A (Horsburgh et al., 2001a) was reported, and a putative quinone oxidoreductase gene was recently discovered in the whole genome sequence of S. aureus (Kuroda et al., 2001).

Although membrane-bound quinone oxidoreductases (Qor) are well recognized in bacteria, few examples of soluble Qors are yet characterized. The membrane-bound Qors such as NADH-ubiquinone oxidoreductase are involved in a respiratory chain (Friedrich, 1998). This enzyme is NADH-dependent, requires either FMN or FAD and may also possess iron–sulfur clusters as cofactors. The soluble Qors are widespread in both prokaryotes and eukaryotes (Persson et al., 1994). The eukaryotic soluble Qors are further divided into two functionally distinct groups, DT-diaphorase and \( \zeta \)-Crystallin. DT-diaphorase is a flavo-enzyme that catalyses an NAD(P)H-dependent two-electron reduction of quinone. This reduction process, which produces hydroquinone, is suggested to involve the detoxification of quinone (Ernster, 1987). On the other hand, \( \zeta \)-Crystallin is a non-flavo-enzyme and catalyses the NADPH-dependent one-electron reduction of quinone to produce the semiquinone radical. This radical is readily oxidized back to quinone in the presence of oxygen, resulting in the generation of superoxide anions. Rao et al. (1992) suggested the involvement of \( \zeta \)-Crystallin in oxidative stress response by mediating hexose monophosphate shunt activity through NADPH homeostasis in the guinea pig. In the plant Arabidopsis thaliana, three \( \zeta \)-Crystallin homologues were identified as genes that complemented the sensitive phenotype to thiol-oxidizing drug diamide in a yeast yap1 mutant that was hypersensitive to oxidative stress (Babiychuk et al., 1995). Mano et al. (2000) reported that one of the three \( \zeta \)-Crystallin homologous genes, Arabidopsis P1, encoded an NADPH : diamide oxidoreductase and demonstrated that the activity was similar to \( \zeta \)-Crystallin. Although these reports suggest that DT-diaphorase and \( \zeta \)-Crystallin play important roles in the oxidative stress response, the catalytic activity and gene expression profile of the bacterial soluble Qors are still unknown.

Because S. aureus is equipped with two SODs (SodA and SodM) (Clements et al., 1999; Valderas & Hart, 2001) and shows a relatively high catalase activity (Yumoto et al., 1999), this organism has a high ability to conquer oxidative stresses. These features of S. aureus are utilized to infect its host. Therefore, it is important to understand oxidative stress response factors of S. aureus and if possible to find a clue to prevent infection by this pathogen. Therefore we paid attention to SA1989, qor homologue, which was identified by N315 genome sequencing analysis (Kuroda et al., 2001). We examined if the product of the qor homologue has Qor activity, and if staphylococcal Qor catalyses a one-electron or a two-electron reduction of PQ (9,10-phenanthrenequinone). Here, we have reported the manner of transcription of the putative qor and its catalytic activity as a Qor. The expression of qor was enhanced under the oxidative stress condition. Moreover, the recombinant protein had an activity as an NADPH-dependent reductase, and superoxide anion generation during PQ reduction. Therefore, it indicates that staphylococcal Qor has a similar activity to \( \zeta \)-Crystallin.

**METHODS**

**Strains and growth conditions.** Escherichia coli was cultured in Luria–Bertani (LB) medium. S. aureus strain N315 was cultured in M9 medium containing 4% glucose, 1% Casamino acids, 1 mM magnesium sulfate and 0.1 mM calcium chloride. Cell cultures were incubated at 37°C with shaking (150 r.p.m.) (TAITEC).

**Materials.** 2-Methyl-1,4-naphthoquinone (menadione) was obtained from Wako and 9,10-phenanthrenequinone (PQ) was purchased from Sigma. 9,10-Diacetoxyphenanthrene (DAP) was synthesized as follows: PQ (0.1 g) was dissolved in 15 ml tetrahydrofuran and then mixed with Zn dust (50 mg), followed by 0.5 ml acetic anhydride, 1 drop water and 3 drops triethylamine. The mixture was stirred at room temperature for 20 min, then an additional 50 mg Zn dust, 1 drop water and 3 drops triethylamine were added and the mixture was heated under reflux for 1 h. After cooling, the mixture was diluted with chloroform (40 ml), washed with water, saturated with NaHCO3 and then dried over MgSO4. The solvent was removed in vacuo to leave a pale yellow solid (0.13 g). The product was recrystallized in benzene and a white solid (45 mg) obtained was identified as DAP by \(^1\)H- and \(^13\)C-NMR, and HPLC/electrospray ionization-mass spectrometry. Acetylated cytochrome C was synthesized as reported previously (Kumagai et al., 2000).

**Preparation of total RNA and Northern blotting.** A 1 ml aliquot of overnight culture of S. aureus was inoculated into 100 ml M9 medium. The exponential-phase culture (OD so 0.5–0.6) was subjected to appropriate shocks. The shocked culture was harvested by centrifugation at 10000 g and 4°C for 1 min. Cells were resuspended in 700 µl 10 mM Tris/HCl (pH 8.0), 1 mM EDTA (pH 8.0) containing 10 µg lysozyme, and were incubated at 37°C for 2 min. Then 1% SDS, 0.3 M sodium acetate (pH 4.8) and an equal volume of phenol (pH 4.8) were added to the cell suspension, followed by five cycles of freezing and thawing (at ~80°C and 65°C). After centrifugation at 10000 g for 10 min at 4°C, the supernatant was ethanol precipitated at ~80°C for 30 min. After washing with 70% ethanol, the pellet was resuspended in 10 mM Tris/HCl (pH 8.0) and 1 mM EDTA (pH 8.0). The obtained total RNA was used for Northern blot analyses and primer extensions. The Northern blots were performed as follows. Total RNA (10 µg) was loaded onto 1% formaldehyde agarose gels and electrophoresed in 1× MOPS buffer. The separated RNAs were transferred to Hybond-N+ (Amersham Biosciences). The blot was pre-hybridized with rapid-hybrid buffer (Amersham Biosciences) at 65°C for 15 min and then a \(^32\)P-labelled DNA probe was added for hybridization at 65°C for 2 h. The hybridized blot was washed with 2× SSC/0.1% SDS at room temperature for 15 min, and with 1× SSC/0.1% SDS and 0.1× SSC/0.1% SDS at 65°C for 15 min each. The blot was autoradiographed with BAS-5000 MAC imaging analyser (Fuji Film). The DNA fragments for probes were amplified by PCR using the chromosomal DNA of N315 as templates.

**Primer extension analysis.** Primer extension reactions were performed by incubating 10 or 20 µg RNA and 5 pmol 5'-FITC-labelled primer (5'-ATCCATTCGAGCACTGCGGTTC-3' for qorA; 5'-ATCCATTCGAGCACTGCGGTTC-3' for SA1990 and 5'-ATCCATTCGAGCACTGCGGTTC-3' for SA1988, respectively) in a reverse transcription mixture (RT mixture) containing 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 1 mM DTT and 0.5 mM dNTPs. In the case of qorA, the RT mixtures were...
incubated at 70°C for 10 min, 55°C for 20 min and 25°C for 10 min. In the case of SA1990 and SA1988, the RT mixtures were incubated at 70°C for 10 min, 58°C for 20 min and 25°C for 10 min. Subsequently, 200 U Superscript II reverse transcriptase (Invitrogen) was added to the RT mixtures, which were then incubated at 42°C for 30 min. The extended products were ethanol precipitated and analysed on 6% urea denaturing polyacrylamide sequencing gels. The upstream regions of the three genes were cloned into pUC119 (Yanisch-Perron et al., 1985) and sequenced with a Thermo Sequenase fluorescence-labelled primer cycle sequencing kit (Amersham Biosciences) using the same oligonucleotides that had been used for the primer extension reactions. Signals were detected with a Fluorolmager image analyser (Amersham Biosciences).

Construction of expression vector for GST–QorA and purification of its product. The gene encoding qorA was amplified from S. aureus N315 (Hiramatsu et al., 1991) by PCR using the following synthetic primers, which were designed to contain the intact Shine–Dalgarno (SD) sequence of qorA: 5’-ATAAATGAAAGCCTTCAATATAA-3’ and 5’-TGAACAAATTTTGCTGCGATAAGA-3’. The PCR product was digested with HindIII and PstI, blunt-ended and cloned into pGEX-2T (Amersham Biosciences) to obtain pGQA, which contains a GST–QorA fusion and an intact QorA expression vector. Strain EQA01 was constructed by transformation of pGQA into E. coli BL21. Strain EQA01 was grown at 37°C overnight in 3 ml LB broth containing 50 µg ampicillin ml⁻¹. Cells were then cultured in 100 ml of the same medium at 37°C and 150 r.p.m. At the exponential phase (OD₆₀₀ 0.5–0.6) of growth, GST–QorA fusion protein was induced by addition of 1 mM IPTG (final concentration). After 2 h, cells were harvested by centrifuging them at 2000 g at 4°C for 10 min. All of the following purification steps were performed at 4°C. Cell pellets were washed with 50 mM Tris/HCl (pH 7.5) containing 5 mM EDTA. After washing, the pellets were resuspended in 50 mM Tris/HCl (pH 7.5) containing 5 mM EDTA and 1 mM PMSF, and were then sonicated twice using a BioRaptor (COSMO BIO) for 3 min each. The cell lysate was then centrifuged at 10,000 g for 20 min. The resulting supernatants were mixed gently with 100 µl Glutathione Sepharose 4B beads (Amersham Biosciences) for 1 h. The beads were collected by centrifugation at 500 g for 5 min. The beads were washed with 50 mM Tris/HCl (pH 7.5) containing 5 mM EDTA and GST–QorA protein was eluted with 10 mM reduced glutathione containing 50 mM Tris/HCl (pH 7.5) and 5 mM EDTA. Thrombin-treated GST–QorA protein was prepared by incubating the bead-bound GST–QorA with 0.5 mg thrombin ml⁻¹ containing 50 mM Tris/HCl (pH 8.0), 150 mM NaCl and 2.5 mM CaCl₂ at room temperature for 1 h, and then the supernatant fractions were collected. To stop the thrombin digestion, 1 mM EDTA was added. The concentration of protein was measured using the Bradford method. Obtained proteins were analysed by 12.5% SDS-PAGE.

Amino acid sequencing. The N-terminal sequencing was done with the protein sequencer ABI 470A (Applied Biosystems). Following SDS-PAGE, the proteins were transferred onto a PVDF membrane. The Coomassie blue stained bands were cut out and applied to the sequencer without Polybrene treatment, as described previously (Ohta et al., 1991).

Measurement of NADPH consumption. The reaction mixture (1 ml) contained the desired concentration of quinone, 0.1 mM NADPH, 50 mM Tris/HCl (pH 7.5), 0.2% Tween-20 and purified enzyme. The reaction was initiated by the addition of quinone. The rate of NADPH oxidation at 25°C over 5 min was monitored by the decrease in absorbance at 340 nm and was calculated by using an extinction coefficient of 6.22 mM⁻¹ cm⁻¹, employing a U-3200 spectrophotometer (Hitachi).

Determination of one-electron reduction of PQ. It is well known that quinones undergo one-electron reduction by enzymes to yield semiquinone radicals which react readily with molecular oxygen to yield superoxide anions (Ernster, 1987). For this reason, the semiquinone radical of PQ was determined by measuring the generation of superoxide anions during reduction of PQ by QorA as described previously (Kumagai et al., 2000). The reaction mixture (1.5 ml) contained 20 µM PQ, 0.1 mM NADPH, 0.1 M potassium phosphate buffer (pH 7.4), 25 µM acetylated cytochrome C and purified enzyme with or without bovine erythrocyte SOD (1000 U). The reaction was initiated by addition of PQ. The rate of reduction of acetylated cytochrome C at 25°C for 5 min was monitored by the increase in absorbance at 550 nm. Superoxide anion generation was calculated as nmol ml⁻¹ of the superoxide dismutase-inhibitable reduction of acetylated cytochrome C by using an extinction coefficient of 21.1 mM⁻¹ cm⁻¹.

Determination of two-electron reduction of PQ. Because 9,10-dihydroxyphenanthrene, a two-electron reduction product of PQ, is a labile catechol metabolite, we determined 9,10-dihydroxyphenanthrene as its diacetoxy derivative. Reduction of PQ to 9,10-dihydroxyphenanthrene was determined as its acetoxo derivative, 9,10-diacetoxyphenanthrene (DAP), by HPLC (K. Taguchi, A. K. Cho, R. Koizumi, N. Shimjo & Y. Kumagai, unpublished). The reaction mixture (1.5 ml) contained 20 µM PQ, 0.1 mM NADPH, purified enzyme and 0.1 M potassium phosphate buffer (pH 7.4), unless otherwise noted. The reaction was initiated by addition of PQ. Incubation was carried out at 25°C for 2 min. A portion of the reaction mixture (0.5 ml) was transferred to a centrifuge tube containing acetic anhydride (20 µl) and heated at 80°C for 5 min: under these conditions, 9,10-dihydroxyphenanthrene formed from PQ was successively converted into DAP. Then the mixture was mixed with 170 µl 10% trichloracetic acid (final 2.5%) and centrifuged at 14,000 g for 5 min. The supernatant (40 µl) was subjected to HPLC analysis. Separation of 9,10-diacetoxyphenanthrene from PQ was performed on YMC-Pack ODS-AM-303 a YMC-Pack ODS-AM (250 × 4.6 mm internal diameter, 5 µm particle size; YMC) with a Shimadzu LC-10AT pump and SPD-10A UV-VIS detector (Kyoto). Elution was accomplished with acetonitrile containing acetic anhydride (20 µl) and heated at 80°C for 5 min: under these conditions, 9,10-dihydroxyphenanthrene formed from PQ was successively converted into DAP. The absorbance at 255 nm. Peak height was determined by a Chromatorecorder 11 (System Instruments). Under these conditions retention times of PQ and DAP were 6.8 and 10.6 min, respectively.

RESULTS

Identification of the qorA gene in S. aureus

As described in the report of whole genome sequencing of S. aureus strain N315, SA1989 was annotated as a putative qor gene (Kuroda et al., 2001) (Fig. 1a). The gene encodes 333 aa. The predicted molecular mass and pI were 36.2 kDa and 5.0, respectively. The hydropathy profile showed the possibility that quinone oxidoreductase (Qor) was not a membrane protein in this case, thus suggesting a cytoplasmic protein. Staphylococcal Qor shared 53% identity with Bacillus subtilis YfmJ, which was also a putative Qor (Kunst et al., 1997), and 18.8% identity with E. coli Qor (Lilley et al., 1993). The Qor in S. aureus contained an NAD(P)H-binding motif, AXXGXXG, as in E. coli Qor (Thron et al., 1995) (Fig. 1b). Because there was no FAD binding motif, this protein would be a non-flavin-containing Qor.
The qor locus in the genome is illustrated in Fig. 1a. In other bacteria qor is unique in the genome, whereas _S. aureus_ possessed another qor homologue (SA1988) just downstream of SA1989. Its product had 26.2% identity with SA1989. The ORF encoded 335 aa. The predicted molecular mass and pI were 37.6 kDa and 5.88, respectively. This protein contained an alcohol dehydrogenase type of NAD(P)H-binding motif sequence, namely, GXGXXG (Persson _et al._, 1994).

Oxidative stress enhances the expression of genes in the qorA operon

When _S. aureus_ establishes an infection in its host, attacks by ROS produced from the host defence systems are lightened by oxidative stress responsive proteins such as SODs and catalase. Available reports of soluble Qors indicate that they are important factors in the response to oxidative stress. Accordingly, to analyse the expression of qorA, a Northern blot analysis was conducted in the presence of the redox-cycling agent, PQ. Signals of 2.6 kb and 1.4 kb were detected, using a qorA gene specific probe, and each signal was clearly enhanced by exposing to PQ in a time-dependent manner. The 2.6 kb signal of qorA was regarded as the transcript co-transcribed with nearby genes such as SA1990 or SA1988 (Fig. 2b). Signals of 2.6 kb were also observed in hybridization with both SA1990 and SA1988 specific probes. In SA1990, two signals (2.6 kb and 1.2 kb) were detected and accumulated by PQ exposure. In SA1988, the 2.6 kb and 1.2 kb signals were also induced by addition of PQ. Besides, a termination structure was identified downstream of SA1988 (Fig. 2b), and loose stem–loops were found downstream of SA1990 and qorA. These results suggested that the qorA gene is expressed as a polycistronic transcript with both SA1990 and SA1988. It should be noted that the expression of lacG, which is located upstream of SA1990, was not enhanced in the presence of PQ (data not shown).

To confirm that these three genes were responsive to oxidative stress, primer extension analyses were done. For qorA, a primer extension signal was strongly enhanced by the addition of PQ (Fig. 3). Although the promoter could be recognized by sigma70, a weak −10 consensus sequence was found 5′ (upstream) of qorA. In the absence of shock, the primer extension signal of SA1990 was not detected, though a signal appeared under the PQ condition, indicating that expression of SA1990 was induced by oxidative stress. There was slight enhancement of primer extension signal in SA1988 with or without PQ exposure. As well as qorA, both SA1990 and SA1988 had an incomplete sigma70 consensus sequence. Despite qorA and SA1990 being inducible by addition of PQ, oxidative regulatory elements such as PerR box or Fur box were not identified in those promoter regions. Northern blot and primer extension analyses revealed that qorA and SA1990 were strongly induced by oxidative stress.

QorA forms a multimer

It has been reported that in _E. coli_ and mammals, Qor and ζ-Crystallin form a homodimer and tetramer, respectively (Thron _et al._, 1995; Huang _et al._, 1987). To investigate if QorA of _S. aureus_ forms a multimer under physiological conditions, an expression vector was constructed. A DNA
A fragment which encoded the full-length \textit{qorA} containing its own SD sequence was inserted into the expression vector for GST-fusion protein, and designated \textit{pGQA}. The vector was transformed into \textit{E. coli} BL21 and designated strain \textit{EQA01}. If the intact \textit{QorA} and GST–\textit{QorA} form a multimer, the former should be co-purified with GST–\textit{QorA}. As shown in Fig. 4, the proteins were obtained from \textit{EQA01} cells by the usual GST purification procedure. Observed after addition of IPTG, two major protein bands of 62 kDa and 36 kDa, which correspond to the calculated molecular mass of GST–QorA and QorA, respectively, were expressed (Fig. 4, lanes 1 and 2, arrows b and d). The fractions eluted from glutathione beads by glutathione solution also contained 62 kDa and 36 kDa proteins (lane 3, arrows b and d). The GST–QorA-conjugated glutathione beads were cleaved by thrombin into GST and QorA, resulting in two bands of 37 kDa and 36 kDa (Fig. 4, lane 4, arrows c and d), and digested GST (Fig. 4, lane 5, arrow e). N-terminal amino acid sequencing confirmed that the 37 kDa and 36 kDa proteins (Fig. 4, lane 4, arrows c and d) contained the N-terminal sequences GSPASN and MQNKQILFNKI, respectively. The N-terminal sequence shown next to arrow c was the sequence of junction between GST and QorA, and that next to arrow d was the \textit{qorA} coding region. These results showed that, in our construct, the products were translated from the SD sequence of GST and the SD derived from staphylococcal QorA, as GST–QorA and QorA were co-purified. Our results clearly demonstrated that \textit{S. aureus} QorA formed a multimer.

**QorA has quinone oxidoreductase activity**

From sequence analysis, the \textit{qorA} gene was predicted to encode a soluble Qor. However, the enzymic activity of soluble Qor has not been reported even in \textit{E. coli}. To investigate whether GST–QorA had NAD(P)H-quinone oxidoreductase activity or not, PQ and 2-methyl-1,4-naphthoquinone (menadione) were used as substrates and NAD(P)H consumption was measured. As shown in Fig. 5, incubation of the purified GST–QorA with 20 µM PQ in the presence of 0·1 mM NADPH resulted in a time-dependent consumption of NADPH (Fig. 5). However, when incubated with menadione, a small amount of NADPH consumption was observed. In contrast, NADH was a poor co-factor for GST–QorA. Such an enzymic activity was not observed with GST, contrasted with GST–QorA.

The kinetic properties of GST–QorA in NADPH consumption were obtained by varying the concentration of substrates (2–100 µM) (Table 1). The \(K_m\) for PQ was

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**Fig. 2.** (a) Northern blots of SA1990, \textit{qorA} and SA1988. (b) Transcripts of the three genes. Boxes and thick lines indicate the genes and specific probes, respectively. Arrows below the boxes show transcripts, sizes and directions. The terminator downstream of SA1988 is indicated by \(T\).
The $V_{\text{max}}$ was $1059 \pm 77\, \text{nmol min}^{-1} \, (\text{mg protein})^{-1}$ for PQ, indicating that the GST–QorA was an active enzyme.

**QorA is capable of a one-electron quinone reduction**

It is well recognized that there are two pathways of quinone reduction by enzymes. One is a one-electron reduction of quinone that generates a superoxide anion. The other is a two-electron reduction of quinone that produces a hydroquinone. To characterize the reducing activity of QorA, we firstly investigated the one-electron reduction of PQ by measuring the generation of superoxide anion. When the purified GST–QorA protein was incubated with PQ in the presence of NADPH, the superoxide anion generation was observed together with NADPH consumption. The amount of superoxide generation in the reaction mixture was $3.42 \, \text{nmol ml}^{-1}$ (Table 2). This value was $53\%$ of NADPH consumption. Such a phenomenon was not observed in the case of GST only (Fig. 6).

Next, two-electron reduction of PQ was evaluated by measuring dihydroxyphenanthrene as its diacetoxy derivative, using HPLC (Fig. 7). If QorA catalyses a two-electron reduction of quinone, hydroquinone should be detected along with NADPH consumption. When GST–QorA (Fig. 7c), but not GST (Fig. 7d), was incubated with PQ in the presence of NADPH, a new peak with a retention time of 10-6 min, corresponding to authentic DAP was detected (Fig. 7a, arrow 2). The small DAP peak was detected immediately after initiating the reaction (0 min) and even

![Image](https://www.microbiologyresearch.org)
without NADPH in the reaction. And its peak did not give the amount of time-dependent increase (data not shown).

Therefore, this small peak is not a product of reduction of PQ by QorA. Besides, the amount of two-electron reduction product of PQ was only 0.27 nmol ml\(^{-1}\) in the reaction mixture (Table 2). This value was just 4% of NADPH consumption. Supplementary data showing this are available at http://mic.sgmjournals.org. Taken together, these results suggested that NADPH-dependent reduction of PQ catalysed by staphylococcal QorA is predominantly the one-electron reduction rather than the two-electron reduction.

**DISCUSSION**

We reported that \(\textit{qorA}\) was induced by oxidative stress, and that the encoded protein acts as an NADPH-dependent, one-electron reductase of PQ, with superoxide anion generation.

All of the three genes, \(\textit{qorA}\), \(\textit{SA1990}\) and \(\textit{SA1988}\), responded to the redox-cycling agent PQ (Fig. 2). Especially strong responses in \(\textit{qorA}\) and \(\textit{SA1990}\) were observed (Figs 2 and 3). Moreover, judging from profiles of expression and sizes, the 2–6 kb signal detected by the \(\textit{qorA}\) probe probably corresponded to two mRNAs, i.e., the co-transcripts of \(\textit{SA1990–qorA}\) and \(\textit{qorA–SA1988}\). Because PQ is one of the substrates for QorA, the enhancement of gene expression of \(\textit{qorA}\) is understandable. As for \(\textit{SA1988}\), since it shares homology with \(\textit{qorA}\), it may also encode one of the quinone oxidoreductases. As regard to \(\textit{SA1990}\), the ORF was 867 bp long and encoded putative polypeptides, similar to a conserved

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**Fig. 4.** SDS-PAGE analysis of the purified proteins. SDS-polyacrylamide gels (12.5%) were stained with Coomassie blue. Whole protein from EQA01 without IPTG (lane 1); IPTG-induced whole protein (lane 2); an eluted fraction from glutathione beads which bound GST fusion proteins (lane 3); a fraction from thrombin-treated glutathione beads which bound GST fusion protein (lane 4); and an eluted fraction from thrombin-treated glutathione beads (lane 5) are shown. Arrows indicate a protein derived from the thrombin solution (a), GST–QorA (b), QorA from thrombin-digested GST–QorA (c), intact QorA (d) and the GST from thrombin-digested GST–QorA (e). The predicted amino acid sequence of the 37 kDa protein (arrow c) is shown in parentheses, and the sequence of the QorA coding region is indicated by an underline.

**Fig. 5.** NAD(P)H consumption by GST–QorA during the reduction of PQ. PQ reduction by GST–QorA with NADPH (●) or NADH (○) is shown. GST was used for the PQ reduction with NADPH (▲). The reaction mixture (1 ml) contained 20 μM PQ, 0.1 mM NADPH, 50 mM Tris/HCl (pH 7.5), 0.2% Tween-20 and the purified enzyme. The reaction was initiated by the addition of PQ. The rate of NADPH oxidation was monitored by the decrease in absorbance at 340 nm.
Table 1. Kinetic constants of NADPH quinone oxidoreductase activity of QorA

Kinetic constants were calculated by using a soft, Analyzing Data with GraphPad Prizm.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_m ) (( \mu M ))</th>
<th>( V_{max} ) (nmol min(^{-1}) (mg protein(^{-1})))</th>
<th>Specific activity (nmol min(^{-1}) (mg protein(^{-1})))</th>
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<tbody>
<tr>
<td>9,10-Phenanthrenequinone</td>
<td>49.1 ± 6.7</td>
<td>1059 ± 77.4</td>
<td>311.0 ± 15.1</td>
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<tr>
<td>2-Methyl-1,4-naphthoquinone (menadione)</td>
<td>ND</td>
<td>ND</td>
<td>14.8 ± 5.2</td>
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*Specific activities were calculated from the values at a concentration of 20 \( \mu M \) substrate.

hypothesized protein. The predicted molecular mass and pl of the SA1990 protein were 32.7 kDa and 10.36, respectively, and it contained 32 lysine residues. The high pl implies that SA1990 is a DNA-binding protein, and it may play a role in the oxidative stress response. This is the first demonstration of oxidative stress induction of the qor gene in bacteria. In S. aureus, either the PerR box or Fur box is responsible for transcriptional activation under oxidative stress (Horsburgh et al., 2001a, b). However, those regulatory sequences were not found at the determined promoter regions of qorA, SA1990 or SA1988 (Fig. 3). This finding indicates the existence of an unknown regulatory system, which remains to be clarified by future studies. To demonstrate the role of qorA under oxidative conditions, we have constructed a qorA disruptant, and measured growth rate and NADPH consumption in the mutant. Compared with the parent strain, however, no significant changes were detected in this mutant (data not shown).

Our construct expressed GST–QorA and intact staphylococcal QorA, which were bound to each other throughout the purification steps. It suggested that staphylococcal QorA forms a multimer under physiological conditions. Other kinds of Qor proteins also form a dimer in E. coli (Thron et al., 1995) and a tetramer in the guinea pig (\( \zeta \)-Crystallin) (Huang et al., 1987). From a crystallization analysis of E. coli Qor, it is revealed that each subunit has an NAD(P)H-binding site, and a quinone binding site, which is thought to be located in a cleft between the co-enzyme and catalytic domains. Edwards et al. (1996) reported that in spite of the low sequence identity between E. coli Qor, Thermoplasma acidophilum glucose dehydrogenase and horse liver alcohol dehydrogenase, these three enzymes shared a three-dimensional structural fold. They also reported that subunit interactions between those enzymes led to a significantly larger change of deviations in catalytic domain and NAD(P)H-binding domain. Because the deviations would ease substrate access, the catalytic activity of the multimer should be higher than that of the monomer. Based on this suggestion, although the three-dimensional structure of QorA is unknown, it can be said that multimer formation may increase the efficiency of activity. A non-redundant structural database search suggested that QorA did not possess FMN or FAD binding motifs. In fact, the oxidation of NADPH in the presence of PQ was not accelerated by the addition of FAD (data not shown).

Quinones are enzymically reduced via two different pathways and the overall pathway is illustrated in Fig. 8. (1) Semiquinone radicals are formed via a one-electron reduction pathway (e.g., NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase, \( \zeta \)-Crystallin, etc.) (Fig. 8, arrow 1), and (2) hydroquinones are formed by a two-electron reduction pathway (e.g., NAD(P)H-quinone oxidoreductase) (Fig. 8, arrow 4) (Ernster, 1987). Semiquinone radicals react with molecular oxygen, resulting in

![Image](https://example.com/image.png)
the generation of superoxide anions (Fig. 8, arrow 2) and lead to redox cycling. As shown in Table 2, hydroquinone formation accounted for less than 10% of superoxide generation during reduction of PQ by GST–QorA. Therefore, staphylococcal QorA predominantly catalyses the one-electron reduction of quinone. In this case, the stoichiometric ratio of NADPH consumption and superoxide anion generation should be 1:2 (Rao et al., 1992). However, the estimated ratio in the present study with QorA was approximately 1:0.5 (Table 2). Theoretically, if the one-electron reduction potential ($E_{1/2}$) of a quinone is below $-155$ mV, which is the $E_{1/2}$ for $O_2/O_2^-$, then the semiquinone radical will undergo redox cycling and generate superoxide anions. However, the $E_{1/2}$ of PQ/PQ$^-$ is $-124$ mV, which is less negative than the $E_{1/2}$ of $O_2/O_2^-$. In the latter, superoxide anions reduce quinone to the semiquinone radical at fairly high rates, resulting in a low steady-state level accumulation of superoxide anions (Cadenas, 1995). Thus, generated superoxide anions would be expected to readily react with PQ and the substantial superoxide anion levels would decrease in the reaction mixture. As a consequence, the PQ radical would be accumulated (Iyanagi, 1987). Alternatively, the accumulated PQ radical may turn to hydroquinone of PQ (Fig. 8, arrow 3).

Despite that staphylococcal QorA mediates generation of superoxide anion, the expression is induced by oxidative stress. Although there has been no obvious contribution of QorA to an oxidative stress response, it remains of interest to identify its role under physiological conditions and oxidative stress in S. aureus.

ACKNOWLEDGEMENTS

We thank Dr William Ba-Thein (Institute of Basic Medical Sciences, University of Tsukuba) for critical reading of the manuscript. This work was supported by grants from the Ministry of Education, Science and Culture of the Japanese Government, and by the Research for the Future Program of the Japan Society for the Promotion of Science (Grant No. 00101411).

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