A homologue to the *Escherichia coli* alkyl hydroperoxide reductase AhpC is induced by osmotic upshock in *Staphylococcus aureus*

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Four major proteins are induced in *Staphylococcus aureus* in response to hyperosmotic shock caused by the presence of two different osmolytes, sucrose and NaCl. The gene encoding one of these proteins was isolated using a novel PCR procedure. The derived protein sequence shows extensive similarity to a subunit of alkyl hydroperoxide reductase (AhpC) from both *Escherichia coli* and *Salmonella typhimurium*. Exposure of *S. aureus* to varying concentrations of H$_2$O$_2$ did not result in the detectable induction of AhpC.

**Keywords**: *Staphylococcus aureus*, alkyl hydroperoxide reductase, osmotic stress, oxidative stress

**INTRODUCTION**

*Staphylococcus aureus* is a pathogen that can cause a wide variety of diseases ranging from superficial cutaneous infections (Noble & White, 1983) to life-threatening systemic diseases (Wheat et al., 1983; Parker, 1984). Certain strains of *S. aureus* are also the causative agents of staphylococcal food poisoning (Bergdoll, 1983; Troller, 1986; Johnson et al., 1991).

*S. aureus* has an unusual characteristic among non-halophilic bacteria in that it has a very low minimum water activity ($a_w$) for growth of 0.86 (Scott, 1953). Integral with the above is the ability of *S. aureus* to tolerate and grow in media or food products containing a high concentration of NaCl (Jay, 1992).

Following extensive studies of osmoregulation in enteric bacteria, there have recently been significant strides in elucidating the physiology of osmoregulation in *S. aureus* (Miller et al., 1991; Graham & Wilkinson, 1992; Townsend & Wilkinson, 1992; Bae & Miller, 1992; Bae et al., 1993; Kaenjak et al., 1993; Pourkomailian & Booth, 1993). The initial effect of osmotic upshock in enterics is the accumulation of K$^+$ induced by a decrease in turgor pressure. *S. aureus*, however, naturally contains high levels of cytoplasmic K$^+$ and glutamate (Kunin & Rudy, 1991; Graham & Wilkinson, 1992), implying that in the event of an osmotic upshock it accumulates some other form of compatible solute. Townsend & Wilkinson (1992) and Bae & Miller (1992) have described the existence of two Na$^+$-dependent proline transport systems in *S. aureus*; a high-affinity system involved in scavenging low concentrations of exogenous proline, and a low-affinity system involved in osmoregulation. Neither system requires induction by proline or by hyperosmotic shock and new protein synthesis is not required for the transport of proline. Hence it seems that osmotic upshock activates a pre-existing system that transports exogenous proline into the cell to act as a compatible solute. The proline transport system and the associated glycine betaine transport systems (Pourkomailian & Booth, 1993) are not therefore candidates for gene induction. The osmo-protective nature of choline with respect to *S. aureus* was first demonstrated by Graham & Wilkinson (1992). Kaenjak et al. (1993) detailed how choline uptake in *S. aureus*, initiated by osmotic stress, occurred via an inducible transport system. Choline utilization is, therefore, one example of the necessity for de novo protein synthesis, and hence gene expression, following osmotic shock. However, to date the induction of genetic systems in terms of osmoregulation in *S. aureus* has not been elucidated.

The present paper initiates such studies and demonstrates that under extreme osmotic stress, caused by two different osmolytes, *S. aureus* induces the expression of a number of novel proteins. One of these proteins is a homologue of...
the *Escherichia coli* *AhpC* component of an alkyl hydroperoxide reductase.

**METHODS**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used were: *Staphylococcus aureus* RN4220 (derivative of NCTC 8325 cured of prophages) (Kreiswirth et al., 1983); *Escherichia coli* JM109 [lac-proAB recA1 endA1 gyrA96 thi bsdR17 supE44 relA1 F' traD36 proAB lacI* {lacZAM151}]) (Yanisch-Perron et al., 1985); *Bacillus subtilis* NCTC 3610; *S. epidermidis* NCTC 11047; *Salmonella typhimurium* NCTC 74; *Listeria monocytogenes* NCTC 4885; *Campylobacter jejuni* NCTC 11351; pUC19 Ap', multicloning site within *lacZ* (Yanisch-Perron et al., 1985). Cultures were grown aerobically at 37 °C, liquid cultures were shaken at 200 r.p.m. Unless otherwise stated, liquid cultures were grown in LB medium (Sambrook et al., 1989) and plated cultures were on LB solidified with 15 g agar 1-1 (LA). Strains were maintained on the stocks at -20 °C. Ampicillin (Ap, 50 mg 1-1), IPTG (40 mg 1-1) and X-gal (40 mg 1-1) (Sigma) were used as required.

**Preparation of total cell protein profiles.** An adaptation of the method described by Johnstone et al. (1982) was used as follows: cultures of *S. aureus* were grown to mid-exponential phase (OD~600~) and 10 ml portions were centrifuged in an MSE-Centrart 2 at 2100 *g* for 15 min. The supernatant was removed and the cell pellet resuspended in 0-7 ml ice-cold PEP buffer [1 mM PMSF (Sigma), 50 mM EDTA (Fisons) made up to volume with PBS (Oxoid)]. The suspension was removed into a glass test tube containing 4-1-4-2 g glass beads (106 pm, Sigma) which was kept on ice. The test tube was vortexed for 1 min intervals, being returned to ice between each interval, until about 90% of the cells had lysed (usually 5-10 min, as determined by phase microscopy on a Nikon Optiphot Microscope). Ice-cold PEP buffer (5 ml) was added to the suspension which was subsequently filtered under vacuum using a 0-45 pm filter (Whatman) to remove the glass beads. The lysed cells suspension was then concentrated using a Centricon-30 device (Amicon, molecular mass cut-off of 30 kDa, maximum vol. 2 ml) or an ultrafiltration unit (Amicon, molecular mass cut-off 30 kDa, maximum vol. 50 ml) depending on the volume of the suspension. The retentate was stored at -20 °C until required.

**Electroblotting of proteins and N-terminal amino acid sequence analysis.** Proteins that were to be subjected to N-terminal amino acid sequence analysis were initially immobilized onto ProBlott PVDF membrane (Applied Biosystems) at 50 V for 30 min at room temperature. After blotting, the membrane was removed from the apparatus, washed in sterile distilled water, dried, and kept in a sterile tube at -20 °C until required. Proteins immobilized onto ProBlott membranes were subjected to N-terminal amino acid sequence analysis performed by J. Keyte and K. Bailey at the Queens Medical Centre, Nottingham, using an Applied Biosystems 473A automatic sequencer.

**Preparation of chromosomal DNA.** Chromosomal DNA was prepared according to the method detailed in Ausubel et al. (1990). During preparation of staphylococcal DNA, cell lysis was achieved via the initial addition of 100 μl lysozymin (10 mg ml-1, Sigma) and 100 μl lysozyme (10 mg ml-1, Sigma) with incubation at 37 °C for 1 h. All bacterial strains were subjected to the treatment of 0-5 ml 10% (w/v) SDS and 50 μl proteinase K (20 mg ml-1, Sigma) with incubation at 37 °C for 1 h. Contaminating proteins and cell wall material were removed by the addition of an equal volume of phenol/chloroform and the DNA was subsequently precipitated by the addition of 0-6 vols 2-propanol. The CsCl purification step was omitted.

**LAB2 Primer.** Amino acid residues 10-16 were chosen from the N-terminal amino acid sequence of Protein 4 as a region that, when reverse-transcribed into DNA, contained the least number of degeneracies; this region, however, still has 1024-fold degeneracy (2 × 4 × 4 × 2 × 4 × 2). This level of degeneracy is too high for application as an effective gene probe and the primer degeneracy was reduced by applying the G-T pairing rule as described by Iwanejko et al. (1989). By this means the degeneracy of the DNA sequence was reduced to eightfold (2 × 2 × 2) as depicted below:

\[
\begin{align*}
5' & \quad \text{TGT-ACG-GCG-CAG-GCT-} \quad \text{TTC-GA} \\
10 & \quad \quad 11 \quad 12 \quad 13 \quad 14 \quad 15 \quad 16 \\
\text{Phe} & \quad \text{Thr} \quad \text{Ala} \quad \text{Gln} \quad \text{Ala} \quad \text{Phe} \quad \text{Asp} \\
\end{align*}
\]

The final primer LAB2 has the additional sequence 5' GGCCC GGAA ATTCA AGCTT3', 5' of Phe codon 10 to provide restriction sites for *Smal*, *EcoRI* and *HindIII*, giving a primer of 40 nucleotides in total.

**PCR technique.** The concept of a novel PCR cloning technique was based on the fact that amplification of the desired gene product would occur without the use of any chromosomal cloning procedures; the only genomic DNA involved would be that of *S. aureus* and problems of hybridization to *E. coli* (see Results and Discussion) could be eliminated.

*S. aureus* chromosomal DNA and pUC19 were digested with *EcoRI* and ligated together. Within this ligation mixture there will be a particular recombinant containing an insert of *S. aureus* DNA on which will be located the gene of interest and hence the complementary sequence to LAB2. This fragment of *S. aureus* DNA could insert into the vector in either of two orientations, i.e. with LAB2 facing *lacZ* (located in the vector DNA) or with LAB2 pointing away from *lacZ*. The whole ligation reaction was then used as the template in a PCR reaction where the primer pairs were either the M13 reverse primer (M13R) and LAB2, or the universal primer (UP) and LAB2.

PCR amplifications were performed as described by Saiki et al. (1988) using 1 U *Taq* DNA polymerase (subsequently renamed Thermostable DNA Polymerase, Advanced Biosystems) per reaction, buffer supplied with the enzyme and 1-5 mM MgCl₂. About 1 μg of each primer per reaction was included and reactions performed in a Technne PHC-3 Thermal Cycler. A typical run involved one cycle of template denaturation at 95 °C for 2 min, followed by annealing of primers to the template at 50 °C for 1 min. Immediately after this, there followed 30 cycles of extension of the primer—template complex at 72 °C for 1 min, denaturation at 95 °C for 30 s and annealing at 50 °C for 30 s. The run finished with an extension at 72 °C for 5 min to ensure completion of all strands. The annealing temperature was varied depending on whether the primers were degenerate and the stringency levels required.

**Plasmid template sequencing.** Sequencing reactions were executed using the Pharmacia T7 Sequencing Kit as per the manufacturer's instructions, with the following modifications: 2 μg (4 μl) template plasmid was denatured by the addition of 2 M NaOH (1 μl) and incubated at room temperature for 10 min. The reaction was neutralized by the addition of 3 M sodium acetate, pH 4-8 (1-5 μl) and sterile distilled water (1-5 μl). Salts were removed by dialysis using 0-025 μm pore filters (VSWPO1300; Millipore) against sterile distilled water for
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30 min. The denatured DNA was made up to 10 μl with sterile distilled water and used in an annealing reaction with annealing buffer (2 μl) and 8.8 ng sequencing primer (2 μl) which was incubated at 37 °C for 20 min. Labelling proceeded as per the Pharmacia instruction booklet with [35S]dATPαS purchased from Amersham.

Polyacrylamide gels (6%, w/v) were used to analyse the sequencing reactions and glass plates were assembled according to the Pharmacia instruction booklet, except that both plates were coated with 5% (v/v) Sigmacote (Sigma)/95% (v/v) chloroform.

Gene sequences and corresponding amino acid sequences were used in homology searches of both DNA (GenBank and EMBL) and protein (Swissprot) databases made available through the Daresbury Seqnet computer system; homology searches were via the FASTA program (Pearson & Lipman, 1988).

**RESULTS AND DISCUSSION**

*Growth of S. aureus at reduced water activity*

*S. aureus* RN4220 was evaluated for growth in TSB (Oxoid) and TSB plus NaCl (0.5–4.0 M), and from the exponential phase of each culture the specific growth rate (μ) was calculated. As the NaCl concentration increased (and thus a_w decreased) the growth rate began to decline from a μ_max of 0.66 to a μ of 0.02 at 3.5 M NaCl; the cell yield also decreased. No growth occurred in media containing more than 3.5 M NaCl even after 30 h. *S. aureus* RN4220 can therefore tolerate between 3.0 and 3.5 M NaCl during aerobic growth, corresponding to an a_w of 0.83–0.86. This is in accordance with previous literature for *S. aureus* (Scott, 1953; Tatini, 1973).

**Protein profiles of osmotically stressed cultures**

SDS-PAGE protein profiles of *S. aureus* cultures were examined after growth in increasing concentrations of NaCl. The production of at least four proteins with molecular masses of about 50 (Protein 1), 40 (Protein 2), 34 (Protein 3) and 25 kDa (Protein 4) were induced at NaCl concentrations of 2.5 M (a_w of 0.88) or above, indicating that *S. aureus* responds to osmotic stress in a manner similar to that observed in the enterics, i.e. hyperosmotic shock causes the induction of a number of proteins. Fig. 1(a) shows a section of a preparative SDS-PAGE gel with a control of total protein extract prepared from non-osmotically shocked cells (lane 2) and total protein extracts from cultures grown in the presence of 2.5 M NaCl (lanes 3 and 4). The technique used to prepare the protein profiles included a concentration step which was achieved by using a Centricon-30 concentrator. This device has a membrane with a molecular mass cut-off of 30 kDa; therefore any proteins with a globular structure and molecular mass above 30 kDa will be retained, while those with a molecular mass below 30 kDa will be lost. Proteins with an unusual conformation, e.g. extended filaments, may show anomalous results. Protein 4 has an estimated molecular mass of about 25 kDa but is still retained by the Centricon-30. In its native state, it must therefore either be a dimer or multimer, have an unusually...
extended conformation, or be a subunit of a larger protein structure, since the concentration step is performed before the proteins are denatured.

To establish that the induction of protein 4 is indeed a consequence of an osmotic stress, the response of *S. aureus* to the alternative osmolyte sucrose was determined. Fig. 1(b) shows the protein profiles of *S. aureus* grown in the presence of 0·6 and 1·2 M sucrose cultures as compared to a control culture containing no sucrose. At least four proteins with molecular masses of about 50 (Protein 1), 40 (Protein 2), 34 (Protein 3) and 25 kDa (Protein 4) were induced in response to osmotic stress caused by 1·2 M sucrose. These are the same molecular masses as the four proteins induced in response to osmotic stress caused by the presence of NaCl (Fig. 1a).

The first 20 residues of Protein 4 were derived following cloning the gene for Protein 4 by reverse genetics to the UP sequence and one at the 5' end within the approximately 600 bp PCR product contains two EcoRI sites, one from the multiple cloning site at the 3' end next to the UP sequence and one at the 5' end within the sequence of LAB2. The fragment was therefore cut with EcoRI and ligated into the corresponding site in pUC19. This construct was used to transform *E. coli* JM109 and a recombinant containing the correct size fragment (pLAB2) was subjected to restriction map analysis and PCR was performed using UP/LAB2 as described in Methods. The primer LAB2 incorporates sites for the restriction enzymes *SalI*, EcoRI and HindIII, and PCR was performed using UP/LAB2 as described in Methods. The DNA sequence (data not shown) of the 624 bp insert was translated for all three reading frames. Only one reading frame contains an ORF of a significant size since the other two reading frames show stop codons at residues 21 and 6, respectively. A comparison of the first 33 bases from the ORF in frame 'a' (ORF1) with the degenerate sequence derived from the N-terminal amino acid sequence showed that they are identical and confirms which bases are used in the positions of degeneracy; thus it is established that pLAB2 contains the DNA coding region for the 190 amino acid 24·3 kDa Protein 4.

Protein 4 shows extensive homology to protein sequences from the databases

A DNA homology search indicated extensive homology between the *S. aureus* sequence from pLAB2 and the *abpC* gene from *E. coli* and *Sal. typhimurium* extending over the majority of the 624 bp (Tartaglia et al., 1990). Since such a high degree of identity exists at the DNA level, a probe designed from any part of the 624 bp PCR fragment will hybridize to the *abpC* region of the *E. coli* genome. This was observed experimentally as the PCR probe appeared to hybridize non-specifically against an *E. coli* host used in preparing a *S. aureus* DNA library (data not shown).

Secondary structure plots

Unidentified ORFs from *B. alcalophilus* (Xu et al., 1991), *Ampibacillus xylanus* (Niimura et al., 1993) and the *AhpC* proteins from *E. coli* and *Sal. typhimurium* (Tartaglia et al., 1990) contain over 50% identity with Protein 4 from *S. aureus* (Fig. 2). In the case of *B. alcalophilus*, the ORF is located adjacent to the *nox* gene which encodes an NADH dehydrogenase (Xu et al., 1991), while in *A. xylanus*, the ORF is adjacent to the *nox* gene, which encodes an NADH oxidase (Niimura et al., 1993). Both enzymes perform similar functions in that they can catalyse electron transfer from NADH to a typical electron acceptor of NADH dehydrogenase and their amino acid sequences share 72·5% sequence identity. Thus, it is reasonable to hypothesize that the two unidentified ORFs upstream, but in the same operon as the *nox* genes, also share similar functions.

AhpC is encoded by *abpC* and this gene is part of an operon in both *E. coli* and *Sal. typhimurium*. The gene immediately downstream from *abpC* is *abpF*, encoding AhpF, and AhpF and AhpC together function as an alkyl hydroperoxide reductase which protects the enteric bacterium against the toxic by-products of oxidative metabolism. Secondary structure comparisons between AhpC from *E. coli*, *Sal. typhimurium* and Protein 4 of *S. aureus*...
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were examined by Chou–Fasman prediction plots (Chou & Fasman, 1978) using the program PLOTSTRUCTURE (Fig. 3). Chou–Fasman prediction plots that appear similar indicate proteins of similar function; these plots indicate the a-helical, /3-sheet, coil regions and /3-turns of a protein and provide a visually illustrative comparison of protein homology that extends beyond primary structure since conservative changes in important amino acids are appropriately reflected. The remarkable identity demonstrated to exist between Protein 4 and the AhpC protein from E. coli and Sal. typhimurium at the gene, amino acid and secondary structure levels indicate that Protein 4 (ORF 1) is the AhpC protein from S. aureus and that pLAB2 contains the coding region of abpC.

Further support for this assignment comes from the observation that a truncated 84 bp potential ORF, 3' to abpC, and in the same reading frame as abpC, has 47.8% identity at the DNA level and 43.5% identity (over 23 amino acids) at the protein level with abpF and its encoded protein AhpF, respectively. AhpF is the large subunit (56 kDa) of the E. coli alkyl hydroperoxide reductase (Tartaglia et al., 1990) and the N-terminal homology to the ORF increases to 65.2% when conservative substitutions are considered (data not shown). It appears that abpC and abpF are adjacent genes in the S. aureus genome, as they are in the enterics (Tartaglia et al., 1990). Interestingly, Protein 1 in Fig. 1(a) has a molecular mass of about 50 kDa, is induced in response to osmotic stress in concordance with Protein 4 and could, therefore, represent the S. aureus AhpF protein. Association of the two alkyl hydroperoxide reductase subunits (namely AhpC and AhpF) would explain the retention of the 25.4 kDa AhpC subunit by the filtration system, which employed a 30 kDa cut-off filter.

In both E. coli and Sal. typhimurium, AhpC is involved in the oxidative stress response; the induction of abpC in response to oxidative stress in S. aureus was therefore investigated.

The induction of S. aureus abpC in response to oxidative stress

Studies on the effects of oxidative stress in Sal. typhimurium (Morgan et al., 1986) and B. subtilis (Murphy et al., 1987), have indicated that concentrations of H2O2 used for pretreatment (60 and 50 pM, respectively) induce the production of oxidative stress proteins within 60 min with no need to subsequently expose cultures to higher concentrations of H2O2 to visualize these proteins. Thus, to examine the protein content of S. aureus under oxidative stress, cultures in mid-exponential phase were subjected to a treatment of 100 pM H2O2 for 10 and 60 min and total cell protein was subsequently prepared and run on an SDS-PAGE gel. Fig. 4 indicates that, using the same protein analysis system (one-dimensional Coomassie-blue-stained gel), no obvious induction of protein occurs within 60 min of exposure to H2O2. Lower levels of
Conclusions

OxyR regulates the induction of AhpC in response to H$_2$O$_2$-induced stress in the enteric bacteria due to oxidation-induced conformational changes in OxyR already bound to an operator site in the promoter region (Christman et al., 1989; Tao et al., 1989; Storz et al., 1990a,b). The OxyR system has not been identified as being functional in stationary phase E. coli cultures which are nevertheless H$_2$O$_2$-resistant, thus it appears that there are two mechanisms by which E. coli can adapt to exposure to H$_2$O$_2$-induced stress, the OxyR regulon and the rpoS (katF; σ$^B$) system; there is no known overlap between the two (Hengge-Aronis, 1993). Whether a similar system occurs in S. aureus, and if so whether it is involved in the response to osmotic shock, remains to be elucidated.

A growth-rate-dependent theory involving an rpoS-like factor is, however, one plausible hypothesis since, as indicated above for E. coli, RpoS (Hengge-Aronis et al., 1993) integrates growth rate induction with the induction of proteins produced in response to both osmotic and H$_2$O$_2$ stress. If in S. aureus, changes in growth rate underlie the osmotic induction of abpC, a hypothesis can be developed which suggests that cultures subjected to a growth-rate-reducing form of stress (e.g. osmotic stress), and which are not also undergoing nutrient starvation, will still metabolize nutrients at the normal rate. Since the growth rate has decreased, the cell is effectively over-metabolizing; the cell senses this over-metabolism in some way and responds by inducing the production of a number of proteins which are globally termed stress proteins. Some of these proteins will help to alleviate the particular form of stress being encountered; in essence a fail safe or ‘safety net’ response. We are currently constructing a strain of S. aureus defective in abpC to establish to what extent AhpC is essential to global stress adaptation in this Gram-positive bacterium.

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