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 H₂O₂ 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; Pourkomaillian & 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 (Pourkomaillian & Booth, 1993) are not therefore candidates for gene induction. The osmoprotective 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

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30 min. The denatured DNA was made up to 10 μ l with sterile distilled water and used in an annealing reaction with annealing buffer (2 μ l) and 8.8 ng sequencing primer (2 μ l) which was incubated at 37 °C for 20 min. Labelling proceeded as per the Pharmacia instruction booklet with [35 S]dATP α S 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 $\mu_{\max}$ 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

Fig. 1. SDS-PAGE gels of *S. aureus* total cell protein extracts prepared from non-osmotically shocked cells and cultures grown in the presence of (a) 2.5 M NaCl (lanes 3 and 4) and (b) 0.6 M sucrose (lane 3) and 1.2 M sucrose (lane 4). 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) are induced (indicated on right-hand side). Lanes S, molecular mass markers; lanes C, uninduced culture control.

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 transfer of the protein onto ProBlott membrane by automatic sequencer computer analysis and the confidence level for all the amino acids named in the sequence is 100 %, the only exception being the initial glycine residue. The information derived from this sequence analysis was utilized to determine the coding region of the structural gene as detailed below.

Cloning the gene for Protein 4 by reverse genetics

The primer LAB2 incorporates sites for the restriction enzymes *Sma*I, *Eco*RI and *Hind*III, and PCR was performed using UP/LAB2 as described in Methods. The approximately 600 bp PCR product contains two *Eco*RI 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 *Eco*RI 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 DNA sequencing of the insert. 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 *ahpC* 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

	1	GLINKEILPF	TAQAFDDPKK	DQFEVTEKD	LKGSWSVCF	YPADFSVCF	50
ORF1		SLINTKIKPF	KNQAF...	KN	GEFIEVTEKD	TEGRWSVEFF	YPADFTFVCF
AhpC (S)		SLINTKIKPF	KNQAF...	KN	GEFIEVTEKD	TEGRWSVEFF	YPADFTFVCF
AhpC (E)		SLINTKIKPF	KNQAF...	KN	GEFIEVTEKD	TEGRWSVEFF	YPADFTFVCF
ORF-Ba				EFIEVSEES	FKGQWSVLCF	YPADFTFVCF	
ORF-Ax		***	***	***	***	*****	***
	51	TELEDLQNY	EELQKLGVDV	FSVSTDTHEV	HKAHSHSDA	ISKITYTMIG	100
ORF1		TELEDVADHY	EELQKLGVDV	YSVSTDTHT	HKAHSSSET	IAKIKYAMIG	
AhpC (S)		TELEDVADHY	EELQKLGVDV	YSVSTDTHT	HKAHSSSET	IAKIKYAMIG	
AhpC (E)		TELEDVADHY	EELQKLGVDV	YSVSTDTHT	HKAHSSSET	IAKIKYAMIG	
ORF-Ba		TELEDLQNEY	AALKELGVEV	FSASTDTHT	HKGWDSSET	IGKITYAMIG	
ORF-Ax		***	***	*****	***	***	***
	101	DPSQTITRNF	DVLDEATGLA	QRGTFIIDPD	GVVQASEINA	DGIGRDASTL	150
ORF1		DPTGALTRNF	DNMREDEGLA	DRATFVDPQ	GIIQAEVTA	EGIGRDASDL	
AhpC (S)		DPTGALTRNF	DNMREDEGLA	DRATFVDPQ	GIIQAEVTA	EGIGRDASDL	
AhpC (E)		DPTGALTRNF	DNMREDEGLA	DRATFVDPQ	GIIQAEVTA	EGIGRDASDL	
ORF-Ba		DPSQTLRNF	DVLNEVSGLA	DRGTFIIDPD	GVVQAEINA	EGIGRDASTL	
ORF-Ax		DPSQTISRQF	DVLNEETGLA	DRGTFIIDPD	GVVQAEINA	DGIGRDASTL	
		**	***	***	***	*****	***
	151	AHKIKAAQYV	RKNPGEVCPA	KWEEGAKT.L	QPGDLVLGKIX		190
ORF1		LRKIKAAQYV	AAHPGEVCPA	KWEEGERLWL	HP.....		
AhpC (S)		LRKIKAAQYV	AAHPGEVCPA	KWEEGERLWL	HP.....		
AhpC (E)		LRKIKAAQYV	AAHPGEVCPA	KWEEGERLWL	HP.....		
ORF-Ba		VNKIKAAQYV	RKNPGEVCPA	KWEEGET.L	KPSLDLVGKI.		
ORF-Ax		INKVKAQYV	RENPEVCPA	KWEEGET.L	KPSLDLVGKI		
		*****	*****	***	***	*****	***

Fig. 2. A matrix of each of the amino acid sequences shown to have significant homology with ORF1 from *S. aureus* was constructed. Identical residues are indicated by an asterisk. Key: AhpC(S), AhpC protein from *Sal. typhimurium*; AhpC(E), AhpC protein from *E. coli*; ORF-Ba, unidentified ORF from *B. alcalophilus*; ORF-Ax, unidentified ORF from *A. xylanus*.

designed from any part of the 624 bp PCR fragment will hybridize to the *ahpC* 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), *Amphibacillus 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 *ndh* 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 *ndh* and *nox* genes, also share similar functions.

AhpC is encoded by *ahpC* and this gene is part of an operon in both *E. coli* and *Sal. typhimurium*. The gene immediately downstream from *ahpC* is *ahpF*, 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*

Fig. 3. Chou-Fasman plots for ORF1 from *S. aureus* (1), AhpC from *E. coli* (2) and AhpC from *Sal. typhimurium* (3) reiterate the extreme structural homology shared by all three proteins. Key: wavy, α -helix; zigzag, β -sheet; straight, coil region; 180° rotation in direction of polypeptide, β -turn.

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 α -helical, β -sheet, coil regions and β -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 *ahpC*.

Further support for this assignment comes from the observation that a truncated 84 bp potential ORF, 3' to *ahpC*, and in the same reading frame as *ahpC*, has 47.8% identity at the DNA level and 43.5% identity (over 23 amino acids) at the protein level with *ahpF* 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 *ahpC* and *ahpF* 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

Fig. 4. SDS-PAGE gel of *S. aureus* total cell protein following oxidative stress with 100 μ M H_2O_2 . Lanes: S, molecular mass markers; C, control (no H_2O_2 treatment); 3, 10 min post-treatment; 4, 60 min post-treatment.

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 *ahpC* in response to oxidative stress in *S. aureus* was therefore investigated.

The induction of *S. aureus ahpC* 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 H_2O_2 used for pretreatment (60 and 50 μ M, respectively) induce the production of oxidative stress proteins within 60 min with no need to subsequently expose cultures to higher concentrations of H_2O_2 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 μ M H_2O_2 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 H_2O_2 . Lower levels of

induction may occur which cannot be deduced from the gel analysis used.

Conclusions

OxyR regulates the induction of AhpC in response to H₂O₂-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 H₂O₂-resistant, thus it appears that there are two mechanisms by which *E. coli* can adapt to exposure to H₂O₂-induced stress, the OxyR regulon and the *rpoS* (*katF*; σ^S) 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 H₂O₂ stress. If in *S. aureus*, changes in growth rate underlie the osmotic induction of *ahpC*, 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 *ahpC* to establish to what extent AhpC is essential to global stress adaptation in this Gram-positive bacterium.

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