Isolation and characterization of *Staphylococcus aureus* starvation-induced, stationary-phase mutants defective in survival or recovery

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Ten *Staphylococcus aureus* mutants, defective in the starvation-induced stationary phase of growth were isolated from two independent Tn917-LTV1 transposon insertion libraries and were designated *suv* as they had apparent survival defects. Seven of these mutants were defective under amino-acid-limiting conditions alone. Two mutants (*suv-3* and *suv-20*) demonstrated lower plating efficiency when starved for glucose, phosphate or amino acids and one mutant (*suv-11*) had reduced plating efficiency after amino acid or glucose starvation. All of the mutants tested were as resistant to hydrogen peroxide assault as the parent, but six were more sensitive to low pH conditions. All the mutants were physically mapped on the *S. aureus* chromosome using PFGE. Chromosomal DNA flanking the Tn917-LTV1 insertion sites was rescued by cloning into *Escherichia coli*. DNA sequence analysis resulted in the identification of a number of transposon-disrupted ORFs encoding putative components such as superoxide dismutase (*suv-1*), haem A synthase (*suv-3*), a component of the SOS response (*suv-9*) and hypoxanthine-guanine phosphoribosyltransferase (*suv-20*). The Tn917-LTV1 insertion created *lacZ* transcriptional fusions for some of the stationary-phase loci. Expression analysis indicated that *suv-4* was induced at mid-exponential phase, whereas *suv-3* and *suv-11* were induced at the onset of stationary phase. The possible roles of these *suv* components in stationary-phase survival or recovery is discussed.

Keywords: *Staphylococcus aureus*, stationary-phase, starvation survival, recovery, insertional mutagenesis

INTRODUCTION

Staphylococcus aureus is a major human pathogen capable of causing a spectrum of diseases ranging from minor infections such as carbuncles to severe sepsis. Localized infections with *S. aureus*, especially in wounds, are very common because of its ability to survive and persist in a wide range of environments such as mammalian abscesses (Nahmias & Shulman, 1972) or dry dust (Lidwell & Lowbury, 1950). The ability to survive outside the host and the difficulty in its eradication has contributed to *S. aureus* being one of the most

important causes of nosocomial infections worldwide (Ayliffe, 1997; Maniatis *et al.*, 1997). The emergence of a new epidemic methicillin-resistant *S. aureus*, designated EMRSA-16, is one of the most intractable problems facing hospital practice today (Communicable Disease Report, 1996). EMRSA-16 is highly transmissible, especially by hand contact and has been implicated in a large number of outbreaks (Communicable Disease Report, 1994). An understanding of how *S. aureus* is able to survive starvation and other environmental stresses might offer opportunities for improvements in the control of this organism as it cycles from host to terrestrial milieus.

Recently, Watson *et al.* (1998) showed that carbon limitation, or multiple-nutrient-starvation, are the most

Abbreviations: CDM, chemically defined medium; MUG, 4-methylumbelliferyl β -D-galactoside.

important stimuli for the development of long-term survival potential in *S. aureus*. Starvation results in decreased cell size and enhanced resistance to acid shock and hydrogen peroxide stress. Long-term survival is dependent on continued protein synthesis and differential expression of proteins has been demonstrated deep into starvation (Watson *et al.*, 1998). These observations are in concordance with previous studies on the starvation-induced stationary-phase survival kinetics of other non-spore-forming bacterial species, including *Micrococcus luteus*, *Vibrio* sp., *Sinorhizobium meliloti*, *Rhizobium leguminosarum* bv. *phaseoli*, *Escherichia coli* and *Salmonella typhimurium* (Jenkins *et al.*, 1988; Kaprelyants & Kell, 1993; Kjelleberg *et al.*, 1993; Matin *et al.*, 1989; O'Neal *et al.*, 1994; Thorne & Williams, 1997; Uhde *et al.*, 1997). There is no evidence for the formation of a viable but non-culturable state in *S. aureus* during starvation as shown by using flow cytometry (Watson *et al.*, 1998).

Analysis of cellular proteins synthesized during starvation has shown that differential protein synthesis occurs. Proteins are temporally expressed throughout different stages of starvation (Lange & Hengge-Aronis, 1991; Nystrom, 1995; Nystrom *et al.*, 1990; Reeve *et al.*, 1984; Spector & Cubitt, 1992; Watson *et al.*, 1998). In *E. coli* and *Sal. typhimurium*, expression of some of these starvation-associated proteins is mediated by regulatory components such as RpoS (O'Neal *et al.*, 1994; Lange & Hengge-Aronis, 1991). RpoS is an alternative sigma factor of RNA polymerase which mediates the expression of genes required for long-term starvation survival. These include genes involved in resistance to environmental stresses, such as oxidation, low pH and the host environment associated with virulence (Cheville *et al.*, 1996; Nickerson & Curtiss, 1997; O'Neal *et al.*, 1994; Seymour *et al.*, 1996; Wilmes-Riesenberg *et al.*, 1997). For example, Nickerson & Curtiss (1997) showed that in *Sal. typhimurium*, RpoS controls the expression of *spv* genes during the initial stages of systemic infection.

Recently, Clements & Foster (1998) have characterized the starvation-recovery mechanism of *S. aureus* and have shown that the addition of either glucose or amino acids alone could not recover cells from glucose-induced starvation. Regrowth occurs only by addition of complex medium or a mixture of amino acids and glucose. The starvation-survival phenotype is quickly lost during recovery to allow cells to resume vegetative growth. This process is temporally regulated and requires differential protein synthesis.

The components required for the development and maintenance of the starvation-induced stationary-phase survival state and recovery in *S. aureus* are unknown. Their identification will allow the elucidation of the survival and recovery mechanisms. Starvation survival and recovery may play an important role in the ability of *S. aureus* to persist inside and outside the host and contribute to its success as a pathogen. This paper describes the identification and characterization of *S. aureus* mutants defective in starvation-induced

stationary-phase survival or recovery using transposon mutagenesis.

METHODS

Bacterial strains. The parental strain used in this study was *S. aureus* 8325-4 (Novick, 1967). *S. aureus* strains were maintained in Tryptic Soya Broth (TSB) (Oxoid) medium with or without 1% (w/v) agar at 37 °C. Antibiotics were added where appropriate. *E. coli* DH5 α was grown in Luria-Bertani (LB) (Oxoid) medium with or without 1% agar at 37 °C. *E. coli* plasmids were selected with 50 μ g ampicillin ml⁻¹.

***S. aureus* starvation conditions.** *S. aureus* strains were grown in an amino-acid-limiting, chemically defined medium (CDM) (Hussain *et al.*, 1991; Watson *et al.*, 1998). Glucose- and phosphate-limiting CDM were prepared by lowering the glucose concentration from 1% (w/v) to 0.1% and phosphate concentration from 0.18 M to 36 mM, respectively. For starvation-induced stationary-phase survival/recovery studies, a 50 ml culture of glucose-, amino-acid- or phosphate-limiting CDM in a 100 ml flask was inoculated from an overnight CDM agar plate to an OD₆₀₀ of 0.01 and incubated with shaking (250 r.p.m.) at 37 °C for 18 h. The cultures were then incubated statically at 4, 25 or 37 °C. Colony-forming ability was determined by serial dilution of samples in PBS and spreading onto CDM agar (1% agar). The number of c.f.u. ml⁻¹ was assessed after incubation at 37 °C for 18 h. All data presented are representative of at least two independent cultures, which showed no more than 10-fold variability between equivalent time points.

Stress resistance assays. Starved cells were prepared by growth in glucose-limiting CDM as described above and then incubated statically for 4 weeks at 25 °C. The cells were then harvested by centrifugation (11000 g, 5 min, RT), washed once in PBS by resuspension and centrifugation (as above) and finally resuspended at 5 $\times$ 10⁵ c.f.u. ml⁻¹ in PBS.

For hydrogen peroxide treatment, cells were resuspended in PBS containing 7.5 mM hydrogen peroxide and then incubated at 37 °C. Samples (0.1 ml) were withdrawn and diluted in PBS containing 10 mg catalase ml⁻¹ and plated onto CDM agar. For acid treatment, cells were resuspended in acidified glucose-limiting CDM (pH 2) (Watson *et al.*, 1998) and then incubated at 37 °C. Samples (0.1 ml) were withdrawn, diluted in PBS and plated on CDM agar.

Creation of Tn917-LTV1 insertion libraries. Two independent Tn917-LTV1 transposon insertion libraries of *S. aureus* 8325-4 were created using the temperature-sensitive vector pLTV1 (Camilli *et al.*, 1990). The plasmid was introduced into *S. aureus* 8325-4 by electroporation (Schenk & Laddaga, 1992) and maintained by growth at 30 °C in TSB containing 5 μ g tetracycline ml⁻¹. To create transposon libraries, *S. aureus* 8325-4 pLTV1 was grown at 37 °C in 100 ml TSB (containing 5 μ g each of erythromycin and tetracycline ml⁻¹) to an OD₆₀₀ of 1.0. An aliquot (5 ml) of the culture was harvested by centrifugation (8000 g, 5 min, 37 °C), resuspended in 100 ml prewarmed (43 °C) TSB (containing 5 μ g erythromycin ml⁻¹) and incubated at 43 °C with shaking (250 r.p.m.) to an OD₆₀₀ of 1.5. An aliquot (5 ml) was removed and inoculated into 100 ml TSB (containing 5 μ g erythromycin ml⁻¹) at 43 °C. The culture was incubated for 18 h at 43 °C with shaking (250 r.p.m.) prior to harvesting by centrifugation (as above), resuspension in TSB containing 15% (v/v) glycerol, snap freezing and storage at -70 °C. Both transposon libraries had approximately 5 $\times$ 10⁸ c.f.u. ml⁻¹ and greater than 95% of cells contained transposon insertions.

Selection of stationary-phase mutants. Aliquots of the transposon libraries were spread onto CDM agar plates to yield approximately 150–200 colonies per plate, after incubation at 37 °C for 18 h. The colonies were replica-plated onto two CDM agar plates and again incubated at 37 °C for 18 h. One CDM plate was incubated at 4 °C for 21 d and the other at 25 °C for 21 d. Our previous studies have shown that *S. aureus* cells survive appreciably longer at 4 °C than at 25 °C in CDM (Watson *et al.*, 1998). As a consequence, the plate stored at 4 °C was the stock plate and the 25 °C plate, the selection plate. After 21 d, the selection plate was replica-plated onto a fresh CDM agar plate and incubated at 37 °C for 18 h. Growth on this plate was then compared with the stock plate. Any colonies absent on the replicate from the selection plate but present on the stock plate were putative stationary-phase survival/recovery mutants (*suw*). These *suw* mutants were then picked from the stock plate and re-tested for their survival/recovery ability. To confirm that the transposon insertion was responsible for the observed phenotype, the transposon was transferred into the parental *S. aureus* 8325-4 background using the general transducing phage ϕ 11 as described by Mani *et al.* (1993). The transductants were tested for erythromycin resistance and the stationary-phase survival/recovery defect.

Molecular biological methods. All molecular biological methods were performed as described by Sambrook *et al.* (1989). Chromosomal DNA from *S. aureus* was isolated using a Qiagen 100/G kit according to the manufacturer's instructions except that bacterial cells were lysed by lyso-staphin (Sigma; 200 μ g ml⁻¹) at 37 °C for 30 min.

Rescue of chromosomal DNA flanking Tn917 insertion sites. The Tn917-LTV1 insertions carry a multiple cloning site, the ColE1 origin of replication and an ampicillin resistance gene (Camilli *et al.*, 1990). Chromosomal DNA from the *suw* mutants was digested with either *Xba*I or *Eco*RI. Both these enzymes restrict the DNA within the transposon multiple cloning site and the nearest restriction site in the chromosomal DNA (Youngman, 1990). The digested DNA fragments were purified using the GeneClean II kit, according to the manufacturer's instructions. The DNA fragments were self-ligated at 1 μ g ml⁻¹ to generate a plasmid containing a fragment of chromosomal DNA flanking the transposon, ColE1 origin of replication and the ampicillin resistance gene. The latter two elements are Tn917-LTV1-derived. These plasmids were transformed into *E. coli* DH5 α and clones selected for ampicillin resistance.

Nucleotide sequence analysis. Rescued chromosomal DNA in the Tn917-LTV1-derived plasmids was sequenced using the Taq DyeDeoxy Terminator Cycle Sequencing Kit and the ABI 373A DNA sequencer according to the manufacturer's instructions (Applied Biosystems). A 25 bp oligonucleotide (5' CTCACAATAGAGAGATGTCACCGTC) complementary to the region upstream of the *lacZ*-proximal end of the transposon was used as sequencing primer. Sequencing data were edited and analysed using the STADEN programs (Staden, 1996). Putative ORFs were screened for similarity to protein sequences in the SWISS-PROT protein sequence database library using the FASTA program (SEQNET facility, Daresbury).

PFGE. PFGE was performed as described by Pattee *et al.* (1990) using the CHEF-DR II system (Bio-Rad). The CHEF gel was run at 6 V cm⁻¹ for 22 h at 14 °C with 10–50 s switching times. Southern blot analysis was performed using a digoxigenin-labelled 8 kb *Sal*I fragment, which is internal to the Tn917-LTV1, as probe (Youngman, 1990).

β -Galactosidase assays. β -Galactosidase assays were performed essentially as described by Youngman (1990). Briefly, 0.5 ml aliquots of cells grown in amino-acid- or glucose-limiting CDM were sampled throughout the growth cycle, pelleted (11000 g, 3 min, RT) and snap frozen at –70 °C for later analysis. The frozen cell samples were thawed, lysed in 0.5 ml AB buffer (100 mM NaCl, 60 mM K₂HPO₄, 40 mM KH₂PO₄) containing 100 ng DNase ml⁻¹, 0.1 % Triton X-100 and 25 μ g lysostaphin ml⁻¹. An aliquot (50 μ l) of each sample was added to 5 μ l 4-methylumbelliferyl β -D-galactoside (MUG) solution (0.4 mg ml⁻¹ in 100 % dimethyl sulphoxide) and incubated for 100 min at 25 °C. The reaction was stopped by addition of 2.5 ml 0.2 M Na₂CO₃. β -Galactosidase activity was determined by fluorescence using a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech) using a range of concentrations of 4-methylumbelliferone as standard. One MUG unit of β -galactosidase activity is defined as the activity that hydrolyses 1 pmol MUG min⁻¹ ml⁻¹ per OD₆₀₀ unit.

RESULTS

Isolation of stationary-phase survival/recovery mutants

S. aureus suw mutants were selected using a screen based on their inability to remain culturable as a colony on amino-acid-limiting CDM agar at 25 °C for 21 d. Approximately 20000 colonies from two independent *S. aureus* 8325-4 transposon libraries were screened using this selection procedure. One hundred and fifty-six putative *suw* mutants were identified after the first round of screening. These together with the parental strain, *S. aureus* 8325-4, were subjected to a secondary screen under the same limiting conditions to prove their veracity. Twenty *suw* mutants, designated SPW 1–20, were identified after secondary screening. Liquid starvation-survival assays for all 20 mutants under amino-acid-limiting conditions confirmed their defects. The liquid starvation-survival assays kinetics are shown in Fig. 1 for ten representative mutants which were further characterized. *S. aureus* 8325-4 incubated at 25 °C showed a total loss of viability after 24 d (>10⁷-fold reduction). In contrast, almost all the *suw* mutants lost culturability between days 11 and 19 (Fig. 1). All mutants showed basically similar stationary-phase survival kinetics.

Phage transduction using phage ϕ 11 was used to transfer the Tn917-LTV1 insertions into the *S. aureus* 8325-4 parental background. Five transductants from each mutant were tested for their stationary-phase properties against the original mutants and *S. aureus* 8325-4. In all cases, erythromycin-resistant transductants had the defect, indicating that the mutant phenotype is co-transduced with the Tn917-LTV1 insertion (results not shown).

Physical mapping of Tn917-LTV1 insertions on the *S. aureus* chromosome

Genomic DNA from each mutant was prepared and digested to completion with *Xba*I or *Eco*RI. Southern blot analysis using a digoxigenin-labelled 8 kb *Sal*I

Fig. 1. Stationary-phase survival/recovery kinetics under amino-acid-limiting conditions. (a) *S. aureus* 8325-4 (●), SPW 1 (□), SPW 2 (△), SPW 3 (▲), SPW 4 (■) and SPW 5 (○). (b) *S. aureus* 8325-4 (●), SPW 9 (■), SPW 10 (▲), SPW 11 (□), SPW 14 (△) and SPW 20 (○). Cultures were grown as described in Methods, then incubated statically at 25 °C. One hundred percent viability corresponds to approximately 5×10^8 c.f.u. ml⁻¹ determined after 18 h (6 h post-exponential) growth at 37 °C prior to transfer to 25 °C.

fragment, which is internal to Tn917-LTV1 (Youngman, 1990), gave a single hybridizing band in each mutant (results not shown). This indicates that each mutant had a single Tn917-LTV1 insertion giving rise to the stationary-phase survival/recovery phenotype.

The chromosomal sites of Tn917-LTV1 insertion were physically mapped using PFGE. PFGE of a *Sma*I restriction digest of the *S. aureus* 8325-4 genome generates 16 fragments ranging in size from 9 to 650 kb (El-Adhami & Stewart, 1997). Tn917-LTV1 DNA contains one *Sma*I restriction site. Therefore, upon restriction digestion of chromosomal DNA of the *su*v mutants an extra *Sma*I fragment will be generated as compared to the *S. aureus* 8325-4 strain. As a consequence, it would be expected that one of the characteristic *Sma*I fragments of *S. aureus* 8325-4 would disappear and be replaced by two smaller fragments.

The sum of the two would be equal to the original fragment into which the transposon had inserted, plus the size of Tn917-LTV1 (8 kb).

CHEF analysis mapped the site of insertion of Tn917-LTV1 in SPW 1, 2, 9, 11 and 14 to the largest fragment, band A (~620 kb) (Fig. 2, Table 1). The combined sizes of the two new resulting fragments were approximately equal to the original size of fragment A (Fig. 2, Table 1). This was confirmed by Southern blotting (results not shown). The transposon insertions in mutants SPW 3, 10 and 20 all mapped to band B (~360 kb) (Fig. 2, Table 1). A 120 kb fragment was the only distinguishable new band in SPW 3 and the remaining ~270 kb band overlapped with fragment E as confirmed by Southern blotting (results not shown). SPW 10 and 20 appear to be closely located on fragment B as the resulting fragment sizes appear to be identical (Table 1). However, it is possible that the same size fragments could result from a transposon insertion at either end of the original *Sma*I fragment. PFGE of SPW 5 and 4 mapped the insertions to fragments E (~270 kb) and I (~120 kb), respectively (Fig. 2, Table 1), as confirmed by Southern blotting (Fig. 2, Table 1 and results not shown).

Stationary-phase survival/recovery properties of the *su*v mutants

Phosphate limitation. The stationary-phase survival/recovery kinetics of the ten *su*v mutants were determined under phosphate-limiting CDM conditions, incubated statically, at 25 °C. Only SPW 3 and 20 exhibited reduced plating efficiency compared to *S. aureus* 8325-4 under phosphate-limiting conditions (results not shown). *S. aureus* 8325-4 loses all viability between days 14 and 16 when starved for phosphate ($>10^7$ -fold reduction) compared to between days 8 and 10 for SPW 3 and 20. At day 8, SPW 3 and 20 had 10- and 4-fold lower apparent viability than *S. aureus* 8325-4, respectively.

Glucose limitation. The stationary-phase survival/recovery kinetics for the ten *su*v mutants were determined under glucose-limiting CDM conditions, incubated statically, at 25 °C. Only SPW 3, 11 and 20 exhibited 10 to 100-fold decreased viability compared with *S. aureus* 8325-4 after 21 d of prolonged starvation (Fig. 3).

Resistance to environmental stress

Hydrogen peroxide. After long-term starvation (4 weeks, 25 °C), none of the mutants were any more sensitive to 7.5 mM hydrogen peroxide than the parental strain, *S. aureus* 8325-4 (results not shown).

Acid stress. Long-term starved cells (4 weeks, 25 °C) were treated with a killing pH of 2 (Watson *et al.*, 1998) and their viability determined. SPW 1, 3, 4, 9 and 11 all exhibited increased sensitivity to acid treatment compared to the parental strain, *S. aureus* 8325-4 (Fig. 4).

Fig. 2. PFGE separation of *Sma*I-digested genomic DNA from various *S. aureus* *suv* mutants. (a) Lanes: 1, *S. aureus* 8325-4; 2, SPW 1; 3, SPW 2; 4, SPW 9; 5, SPW 4; 6, SPW 5; 7, SPW 11; 8, λ ladder. (b) Lanes: 1, *S. aureus* 8325-4; 2, SPW 3; 3, SPW 20; 4, SPW 10; 5, SPW 14; 6 λ ladder. The black boxes highlight the digested fragment compared to *S. aureus* 8325-4.

Table 1. Physical mapping of the Tn917-LTV1 insertion site in *suv* mutants

All sizes are approximate under the PFGE conditions used.

Strain	<i>Sma</i> I fragment containing Tn917-LTV1 (kb)*	New fragments (kb)†
SPW 1	A 620	410, 210
SPW 2	A 620	400, 200
SPW 3	B 360	120
SPW 4	I 120	70, 60
SPW 5	E 270	225, 65
SPW 9	A 620	430, 180
SPW 10	B 360	200, 145
SPW 11	A 620	400, 200
SPW 14	A 620	420, 200
SPW 20	B 360	200, 145

* *Sma*I fragments missing due to insertion of Tn917-LTV1 and creation of a novel *Sma*I site.

† Two new *Sma*I fragments generated after Tn917-LTV1 insertion as revealed by ethidium bromide staining. The sizes of these fragments were confirmed by Southern blot analysis using the Tn917-LTV1 as probe.

Fig. 3. Stationary-phase survival/recovery kinetics under glucose-limiting conditions. *S. aureus* 8325-4 (●), SPW 3 (■), SPW 11 (□) and SPW 20 (○). Cultures were grown as described in Methods, then incubated statically at 25 °C. One hundred percent viability corresponds to approximately 5×10^8 c.f.u. ml⁻¹ determined after 18 h (6 h post-exponential) growth at 37 °C prior to transfer to 25 °C.

SPW 3 was the most sensitive, being completely killed after only 10 min treatment.

Table 2 summarizes all the stationary-phase phenotypic studies for the various mutants.

Expression analysis of Tn917-LTV1 *lacZ* transcriptional fusions

Tn917-LTV1 contains a promoterless *lacZ* gene, so if insertion occurs in the correct orientation downstream of a chromosomal promoter a transcriptional *lacZ* fusion is created. Initial expression analysis of the ten

Fig. 4. Acid (pH 2) resistance of the *suv* mutants. *S. aureus* 8325-4 (●), SPW 1 (■), SPW 3 (□), SPW 4 (○), SPW 9 (▲) and SPW 11 (△). Cells were starved under glucose-limiting conditions for 28 d prior to acid treatment as described in Methods. The percentage survival was calculated by plating. One hundred percent viability corresponds to approximately 5×10^8 c.f.u. ml⁻¹. The results are representative of at least two independent experiments.

suv mutants indicated that only three (SPW 3, 4 and 11) had detectable levels of β -galactosidase activity. SPW 4 exhibited the highest β -galactosidase activity of approximately 28 and 36 MUG units for amino-acid- and glucose-limiting conditions, respectively, during exponential phase. This activity did not increase in stationary phase (Fig. 5a). SPW 3 showed a peak of expression as the cells entered stationary phase of 18 and 25 MUG units for amino-acid- and glucose-limiting conditions, respectively (Fig. 5b). Expression of SPW 11 also showed a stationary-phase peak of 14 and 11 MUG

units for amino acid and glucose limitation, respectively (Fig. 5c).

Sequence analysis

To identify the *suv* genes, the chromosomal DNA flanking the Tn917-LTV1 insertion sites was rescued. DNA sequence analysis was carried out to determine the nature of the *suv* genes. Approximately 300 bp of chromosomal DNA sequence proximal to the Tn917-LTV1 insertion site for each mutant was generated. Database searches revealed five of the *suv* genes to have homology to known sequences which may shed light on their possible roles (Table 3). The mutated genes in SPW 2, 5, 10, 11 and 20 showed no significant homology to known proteins in the database.

DISCUSSION

S. aureus mutants defective in starvation-induced stationary-phase survival/recovery responses were isolated using a combination of transposon mutagenesis and a screening technique based on the inability of the mutants to remain culturable under amino-acid- and nutrient-limiting conditions. The screen is such that it will identify components important in either long-term survival or recovery as both will result in a reduction in plating efficiency. Southern blot analysis revealed that only one copy of the transposon had inserted into the chromosome of each of the *suv* mutants. Phage transduction was used to show that in all cases the stationary-phase survival/recovery phenotype was transferred with the Tn917-LTV1 insertion.

All of the mutants isolated had 10- to 100-fold lower plating efficiency compared to the parental strain, *S. aureus* 8325-4. This observation is in accordance with the drop in viability observed in starvation-induced

Table 2. Summary of stationary-phase survival studies

—, No defect as compared to *S. aureus* 8325-4; +, defective as compared to *S. aureus* 8325-4.

Mutant	Survival defect under nutrient limitation			Environmental stress resistance	
	Amino acid	Phosphate	Carbon	Acid sensitivity	Oxidative stress sensitivity
8325-4 (WT)	—	—	—	—	—
SPW 1 (<i>suv-1</i>)	+	—	—	+	—
SPW 2 (<i>suv-2</i>)	+	—	—	—	—
SPW 3 (<i>suv-3</i>)	+	+	+	+	—
SPW 4 (<i>suv-4</i>)	+	—	—	+	—
SPW 5 (<i>suv-5</i>)	+	—	—	—	—
SPW 9 (<i>suv-9</i>)	+	—	—	+	—
SPW 10 (<i>suv-10</i>)	+	—	—	—	—
SPW 11 (<i>suv-11</i>)	+	—	+	+	—
SPW 14 (<i>suv-14</i>)	+	—	—	—	—
SPW 20 (<i>suv-20</i>)	+	+	+	+	—

Fig. 5. Expression of *suv* genes. Strains were grown in CDM limiting for either glucose or amino acids at 37 °C. Growth was determined by OD₆₀₀ and *suv* expression by β-galactosidase activity as described in Methods. (a) SPW 4: OD₆₀₀ in glucose-limiting CDM (□) and in amino-acid-limiting CDM (○); β-

stationary-phase survival/recovery mutants isolated in other bacterial species. For example, in *Vibrio* sp., the starvation-survival mutant *csrS* exhibited a 100-fold lower viability compared to the parental strain after 8 d of carbon starvation (Ostling *et al.*, 1995). Similarly, in *Sal. typhimurium*, three mutants designated *stiA*, *stiB* and *stiC*, exhibited 45- to 70-fold lower viability than the parent after 20 d starvation (Spector & Cubitt, 1992). In *E. coli*, a mutation in the *pcm* gene, encoding L-isoaspartyl protein methyltransferase, results in a 10-fold drop in viability as compared to the parent after 10 d of glucose starvation (Li & Clarke, 1992). In addition, a mutation in the *rpoS* gene, encoding a sigma factor, exhibits a 100-fold lower viability than the parent after 8 d of starvation (Lange & Hengge-Aronis, 1991).

The *suv* mutants isolated in this study could be separated into three groups by virtue of their phenotypes (Table 2). Seven of the mutants (SPW 1, 2, 4, 5, 9, 11 and 14) exhibited a defect under amino-acid-limiting conditions alone, one (SPW 11) exhibited reduced plating efficiency during glucose or amino acid starvation and two (SPW 3 and 20) had lowered survival potential when starved under glucose, phosphate or amino-acid-limiting conditions. The *suv-3* and *suv-20* genes may encode proteins required for maintenance of viability irrespective of the starvation stimulus.

Long-term glucose starvation in *S. aureus* has been shown to elicit a cross-protection to acid stress (Watson *et al.*, 1998). Several of the *suv* mutants showed increased acid sensitivity after long-term stationary-phase survival. This result is not surprising as the mutants were identified under amino-acid-limiting conditions. The pH of the supernatant of an amino-acid-limiting medium upon starvation was found to decrease from 7.0 to 5.5 (Watson *et al.*, 1998). This acidification of the medium might contribute to the selection of acid-defective mutants as observed in the six *suv* mutants. Starvation-associated cross-protection against environmental stress is characteristic of many bacteria and allows the organism to survive environmental assault over a period in which it cannot rapidly adapt (Cheville *et al.*, 1996; Nickerson & Curtiss, 1997; O'Neal *et al.*, 1994; Seymour *et al.*, 1996; Wilmes-Riesenberg *et al.*, 1997).

Several workers have used *lacZ* transcriptional fusions to isolate starvation-induced genes and consequently identify starvation-survival and recovery loci (Lange & Hengge-Aronis, 1991; Spector & Cubitt, 1992; Uhde *et al.*, 1997). Only three of the *suv* mutants (SPW 3, 4 and

galactosidase activity in amino-acid-limiting CDM (●) and in glucose-limiting CDM (■). (b) SPW 3, OD₆₀₀ in glucose-limiting CDM (□) and in amino-acid-limiting CDM (○); β-galactosidase activity in amino-acid-limiting CDM (●) and in glucose-limiting CDM (■). (c) SPW 11, OD₆₀₀ in glucose-limiting CDM (○) and in amino-acid-limiting CDM (□); β-galactosidase activity in amino-acid-limiting CDM (■) and in glucose-limiting CDM (●). The results are representative of at least two independent experiments.

Table 3. Homology of the proteins predicted from the primary amino acid sequence and their function

Mutant	Homology				
	Species	Accession no.	Gene	Identity (%)*	Homologue
SPW 1	<i>S. epidermidis</i>	X97011	<i>sodA</i>	88	Superoxide dismutase
SPW 3	<i>B. subtilis</i>	P12464	<i>ctaA</i>	57	Haem A synthase
SPW 4	<i>B. subtilis</i>	P12464	<i>rpoE</i>	38	δ -Subunit RNA polymerase
SPW 9	<i>B. subtilis</i>	Z99115	<i>umuC/samB</i>	54	SOS response component
SPW 20	<i>B. subtilis</i>	P37472	<i>hprT</i>	55	Hypoxanthine-guanine phosphoribosyltransferase

* Refers to the deduced amino acid sequence over a minimum of 54 deduced residues.

11) had the transposon-insertion-derived *lacZ* in the correct orientation, or expressed it at high enough levels to measure- β -galactosidase activity. The expression of *suv-4* was induced at mid-exponential phase, whereas *suv-3* and *suv-11* were induced as the cells entered stationary phase. The *suv-4* transcriptional fusion exhibited phase 0 loci induction kinetics, whereas the *suv-3* and *suv-20* exhibited phase 1 loci induction kinetics as observed for starvation-stress response genes in *Sal. typhimurium* (Spector & Cubitt, 1992). This correlates with *suv-3* and *suv-11* playing a role in long-term stationary-phase survival/recovery under both amino acid and glucose limitation.

Analysis of the chromosomal DNA sequence flanking the Tn917-LTV1 insertion sites resulted in the identification of a number of transposon-disrupted ORFs. Comparison of the Suv proteins with sequences in the database demonstrated several similarities to known proteins (Table 3).

SPW 4 has a mutation in a gene homologous to *rpoE* of *Bacillus subtilis* (38.5% over 75 aa). RpoE is the δ subunit of RNA polymerase which binds to the core enzyme and also its different holoenzyme forms, thereby inhibiting transcription of non-specific templates in *B. subtilis* (Tjian *et al.*, 1977). Recently Desaro *et al.* (1995) showed that RNA polymerase purified from a *B. subtilis* mutant with an insertion in the δ gene contains a truncated δ protein, indicating that the amino-terminal domain is stable *in vivo* and contains the core-binding function. This suggested that the function of the amino-terminal domain of δ protein is to bind and orientate the carboxyl-terminal region on the surface of RNA polymerase (Desaro *et al.*, 1995). In SPW 4, the Tn917-LTV1 insertion occurred within the putative amino-terminal domain of the δ protein. Expression analysis of the putative *S. aureus* *rpoE::lacZ* transcriptional fusion (strain SPW 4) increased during mid-exponential phase, suggesting that *suv-4* may play a regulatory role in stationary phase.

SPW 9 has Tn917-LTV1 inserted in a gene with strong similarity to a protein involved in the SOS response in *B. subtilis* (54% over 79 aa), designated UmuC, SamB or MucB depending on its origin (Walker, 1995). Exposure

to conditions that damage DNA, such as UV-radiation, results in the induction of more than 17 genes, termed the SOS response (Walker, 1984). The *umuC* gene is part of the *umuCD* operon (Shinagawa *et al.*, 1983). Upon SOS induction, UmuC interacts with several proteins, including the post-transcriptionally modified UmuD, the activated form of RecA and DNA polymerase II holoenzyme, such that an error prone repair DNA lesion that normally blocks DNA replication is overcome (Walker, 1995). DNA repair systems are crucial to the viability of long-term starved cells, since a DNA replication blockage will prevent recovery from starvation.

During stationary phase and long-term starvation, nutrient scavenging and salvaging systems are induced. SPW 20 has an insertion in a gene homologous to *hprT* from *B. subtilis* (55% over 90 aa). Hypoxanthine-guanine phosphoribosyltransferase is a key enzyme in the salvaging system for purine nucleotides. This salvage pathway comprises a collection of enzymes capable of converting nucleobases and nucleosides to nucleotides (Endo *et al.*, 1983; O'Reilly *et al.*, 1984). This enables organisms to use exogenous bases liberated from lysed cells or recycle those produced by the breakdown of unstable RNA. The purine salvage pathway is important during starvation, both as a source of energy and for the maintenance of purine pools. Hypoxanthine is the predominant intracellular nucleobase during stationary phase in *E. coli* (Rinas *et al.*, 1995). SPW 20 exhibits a stationary-phase survival/recovery defect under glucose, amino acid and phosphate limitation. Thus, *suv-20* plays an important role in stationary phase irrespective of the limiting nutrient.

The oxidative stress resistance is important for the survival of *S. aureus* (Watson *et al.*, 1998). Reactive oxygen species cause severe damage to macromolecular cellular components, including RNA, membrane, proteins and most importantly DNA (Fridovich, 1997). One of the defences of the cell against oxidative stress is the synthesis of enzymes, like superoxide dismutase and catalase, that directly destroy reactive oxygen species and thus prevent oxidative damage. The importance of catalase during starvation was highlighted by the initial isolation of the starvation-survival sigma factor, σ^S , as a

catalase-deficient mutant (Loewen & Triggs, 1984). SPW 1 carries an insertion in a gene encoding a protein with a strong similarity to the Mn-SOD family in *Staphylococcus epidermidis* (88% over 54 aa). Considering the importance of oxidative stress during starvation it is unsurprising that a strain with lowered SOD activity is also a stationary-phase mutant. Three types of SOD have been identified in eubacteria: Mn-SOD (Touati, 1988), Fe-SOD (Yost & Fridovich, 1973) and Cu-Zn-SOD (Imlay & Imlay, 1996). A Cu-Zn-SOD has been shown to be necessary for starvation survival in *Legionella pneumophila* (St. John & Steinman, 1996).

During starvation, metabolism will alter to reflect nutrient availability and energy requirements of the cell. The use of different terminal oxidases, which are involved in electron transfer, allows the cell to modify its bioenergetics, maximizing energy production in a constantly changing environment. Significant variation in the composition and organization of the *B. subtilis* electron transport chain is observed in response to the environment. The transposon insertion in SPW 3 inactivated a gene encoding a protein with a strong similarity to CtaA of *B. subtilis* (57% over 56 aa). Deletion of the *ctaA* gene in *B. subtilis* results in the total lack of membrane *a*-type cytochromes (*aa₃* and *caa₃*) and an inability to sporulate (Mueller & Taber, 1989). It is suggested that *ctaA* encodes a haem A synthase, possibly haem O monooxygenase and/or deoxygenase (Svensson & Hederstedt, 1994). The inactivation of the haem biosynthetic enzyme (CtaA) in *S. aureus* might result in the loss of a starvation-specific terminal oxidase. This major disruption of cell metabolism may lead to either a loss of viability during starvation or the inability to recover from stationary phase. As in *B. subtilis* (Van der Oost *et al.*, 1991), expression analysis of the putative *S. aureus ctaA::lacZ* transcriptional fusion (strain SPW 3) showed that *ctaA* transcription increased at the onset of stationary phase in both amino-acid- and glucose-limiting conditions. This is consistent with SPW 3 being a stationary-phase survival/recovery mutant under amino acid, phosphate and glucose limitation and demonstrates the similarity between CtaA of both *B. subtilis* and *S. aureus*.

Recently, Vasilieva *et al.* (1997) isolated a *ctaA* mutant of *S. aureus* which shows an increased resistance to cefoxitin, a β -lactam. This mutant also forms a small colony variant similar to that seen in persistent and antibiotic-resistant *S. aureus* infections (Proctor *et al.*, 1996). *S. aureus* small colony variants are associated with decreased haemolytic activity (α -toxin), decreased coagulase activity, reduced pigmentation and slow growth. These characteristics allow *S. aureus* small colony variants to persist within host cells and resist antimicrobial agents (Proctor *et al.*, 1996). Whether or not the role of CtaA in systemic infection is enhanced by its stationary-phase survival/recovery attributes remains to be proven.

Our work has identified a number of loci important in stationary-phase survival/recovery of *S. aureus*. Our current research is aimed at determining the molecular

basis of the role of these components and how they are regulated during the life cycle of *S. aureus*.

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