Starvation survival in *Listeria monocytogenes*: characterization of the response and the role of known and novel components

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The starvation survival response (SSR) of *Listeria monocytogenes* EGD is induced under glucose- or multiple-nutrient-, but not amino-acid limitation. 0.01–0.2% of the population remain viable even after 20 d and the survivors show a reduced cell size and increased cross-protection to several environmental stresses. The development of the SSR may therefore be important in *L. monocytogenes* survival in the food environment. The initiation, but not the maintenance, of the SSR involves both protein and cell wall biosynthesis. It is also likely that nutrients released from dead cells are recycled to allow survival of the remaining population. To define the molecular mechanisms involved in the initiation, maintenance and release from the SSR the role of known, and novel, components was examined. The well-characterized regulators SigB and PrfA are both required for the full SSR and effect stress resistance during growth and starvation. A transposon mutagenesis screen identified two novel loci with roles in the SSR and stress resistance. Characterization of the transposon insertion sites revealed a putative homologue of the gene *yulB* from *Bacillus subtilis* and a gene of unknown function. The potential individual and combined roles of the SSR components are discussed.

Keywords: stress resistance, nutrient limitation, SigB, PrfA

INTRODUCTION

In the natural environment, fluctuations in the availability of nutrients are common. To cope with lengthy periods of nutrient limitation, bacteria have developed a starvation survival response (SSR). This involves a physiological adaptation to insufficient nutrients for growth. The SSR has been well studied in a number of species including *Vibrio* spp., *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus* (Foster & Spector, 1995; Kjelleberg *et al.*, 1993; Kolter *et al.*, 1993; Watson *et al.*, 1998a).

During starvation, most of the population of *S. aureus* dies; however, the survivors undergo a differentiation process resulting in smaller cells (Watson *et al.*, 1998b). Associated with long-term starvation, many species show an increase in resistance to a number of en-

vironmental stresses such as low pH, heat and oxidative stress (Hartke *et al.*, 1994; Watson *et al.*, 1998a). Development of the SSR is dependent on differential gene expression (Morton & Oliver, 1994; Reeve *et al.*, 1984), and a number of components important in its development and maintenance have been characterized (Cashel *et al.*, 1996; Hengge-Aronis, 1996; Watson *et al.*, 1998b). Important in the instigation of general stress resistance in Gram-positive bacteria such as *Bacillus subtilis*, *Listeria monocytogenes* and *S. aureus* is the alternative sigma factor SigB, although its role in the SSR is unclear (Becker *et al.*, 1998; Chan *et al.*, 1998; Hecker & Völker, 1998).

L. monocytogenes is well known as a food-borne pathogen, particularly associated with outbreaks involving dairy and meat products (Farber & Peterkin, 1991). Infection in humans can occur as bacteraemia, meningitis or disseminated infection, and is especially notable for the high mortality rate it causes in neonates (10–50%) (Farber & Peterkin, 1991). The regulation of virulence gene expression is known to occur in response

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Abbreviation: SSR, starvation survival response.

to environmental signals, and is governed by the transcriptional activator PrfA (Behari & Youngman, 1998; Böckmann *et al.*, 1996; Leimeister-Wächter *et al.*, 1992; Mengaud *et al.*, 1991). The bacterium is highly versatile, and can be found in a remarkably wide range of environments, including water, soils, silage, plant surfaces and the human intestinal tract. It is likely that this organism commonly encounters conditions of starvation, and thus the SSR of *L. monocytogenes* has implications for survival of the bacterium in the environment, in the colonization of food production sites, in the subsequent colonization of food products and in the ability to establish infections. Survival of *L. monocytogenes* has been studied in foodstuffs; however this is typically in relation to food preservation methods (Bolton & Frank, 1999; Casadei *et al.*, 1998; Gahan *et al.*, 1996). In this work we have characterized the SSR of *L. monocytogenes* EGD, and have identified the roles of two novel starvation survival loci.

METHODS

Bacterial strains. Strains of *L. monocytogenes* used in this study are shown in Table 1. Strains were routinely maintained on TSB agar (Difco Tryptic Soy Broth with 1.5%, w/v, Oxoid Agar no. 1), and plates were incubated at 37 °C. Glycerol stocks of strains were kept at -20 °C for long-term storage.

Growth and starvation of bacterial strains. *L. monocytogenes* serovar 1/2a, strain EGD was grown in glucose-limited chemically defined medium (CDM) unless otherwise stated. CDM was modified from that described by Trivett & Meyer (1971). The composition of glucose-limited CDM was as follows (per litre): NaOH, 0.24 g; nitrilotriacetic acid, 0.48 g; FeCl₃, 0.048 g; MgSO₄·7H₂O, 0.41 g; NH₄Cl, 1.0 g; K₂HPO₄, 0.85 g; NaH₂PO₄, 0.15 g; MOPS, 4.18 g; L-lysine.HCl, 0.1 g; L-leucine, 0.1 g; DL-isoleucine, 0.2 g; DL-valine, 0.2 g; DL-methionine, 0.2 g; L-arginine, 0.2 g; L-histidine, 0.2 g; glucose, 1.0 g; α -lipoic acid, 10 μ g; biotin, 1 mg; thiamin, 5 mg; riboflavin, 1 mg. The liquid medium was prepared as described by Trivett & Meyer (1971) excepting: (i) K₂HPO₄ and NH₄Cl were dissolved in only 480 ml distilled water; (ii) 20 ml sterile 1 M MOPS (pH 7.5) solution was added to the final medium; (iii) the amino acids were dissolved in 390 ml distilled water.

Glucose- or amino-acid-limited CDM was made by decreasing the glucose concentration from 0.2% (w/v) to 0.1% or

reducing the total amino acid concentration from 0.12% (w/v) to 0.0016%, respectively. Glucose-minus medium was prepared by omitting glucose from the medium.

Starvation cultures were inoculated to an OD₆₀₀ of 0.01, prepared as previously described (Watson *et al.*, 1998b), incubated with shaking (250 r.p.m.) at 37 °C to stationary phase (18 h), then incubated statically at 25 or 37 °C. All cultures reached an OD₆₀₀ of 0.65–0.75, which corresponded to approximately 1×10^9 c.f.u. ml⁻¹. Multiple-nutrient-limited cells were prepared by harvesting stationary-phase CDM cultures (37 °C, 18 h) by centrifugation (4000 g for 10 min at room temperature). The cells were washed twice by resuspension and centrifugation with an equal volume of PBS (Sambrook *et al.*, 1989) or distilled water, before being resuspended in PBS or distilled H₂O, respectively, to the original culture volume. Viable counts were determined by serial dilution in PBS, plating on TSB agar and incubation overnight at 37 °C. The minimum detection level was 100 c.f.u. ml⁻¹. Results are mean values of at least two separate experiments, for which the SD was less than $\pm 40\%$ of the mean.

Electron microscopy. Samples were centrifuged (10000 g for 5 min at 4 °C) and the supernatant removed. The cells were fixed and stained by standard means (Watson *et al.*, 1998b), and sections studied with a Phillips CM-10 transmission electron microscope. Mean values $\pm$ SD were calculated from at least 60 individual cells viewed within a randomly selected area.

Penicillin G. Penicillin G to a final concentration of 20 μ g ml⁻¹ (20 $\times$ MIC) was added to starved cultures incubated at 37 °C. Penicillin G was still biologically active to greater than MIC level after 15 d incubation at 37 °C (data not shown). Glucose-limited CDM was used for all penicillin G experiments.

Chloramphenicol. Synchronous glucose limitation of cultures was achieved by harvesting mid-exponential (OD₆₀₀ 0.3, 37 °C) cells by centrifugation and resuspension in glucose-minus CDM. Chloramphenicol (100 μ g ml⁻¹) was added after 0, 1, 2, 4, 8 and 24 h incubation at 37 °C. Control cultures with no chloramphenicol added were also monitored. Viability was determined by plating on TSB agar.

Stress resistance. Mid-exponential-phase and 6 h post-exponential-phase cells grown at 37 °C were harvested by centrifugation (5000 g, 3 min, room temperature). Challenge with heat, acid or oxidative stress was carried out as described by Watson *et al.* (1998a), except that acid resistance was examined in CDM adjusted to pH 3.5 with HCl. Viability was determined by plating on TSB agar.

Table 1. *L. monocytogenes* strains used in this study

Strain	Relevant genotype	Parent strain	Source
EGD	Wild-type	–	Wuenschel <i>et al.</i> (1993)
LMA2B	<i>sigB</i> , Km ^R	10403S	Becker <i>et al.</i> (1998)
DES011	<i>sigB</i> , Km ^R	EGD	This study
DES012	<i>prfA</i>	EGD	C. Dickneite*
DES028 (DES029)	<i>yulB</i> : : Tn917	EGD	This study
DES035 (DES045)	ORF1 : : Tn917	EGD	This study

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Phage transduction of mutations into wild-type EGD background. Lysates of *L. monocytogenes* LMA2B carrying a null mutation of the *sigB* gene (Becker *et al.*, 1998) and starvation-survival-defective mutants were prepared using bacteriophage ϕ LMUP35 (Hodgson, 2000). Exponential-phase cells of *L. monocytogenes* EGD (1×10^8 c.f.u.) were incubated for 40 min at room temperature with 1×10^8 p.f.u. of phage. The mixture was overlaid onto BHI agar (Difco) plates containing sodium citrate (10 mM, pH 7.5) and kanamycin ($40 \mu\text{g ml}^{-1}$) using 3 ml BHI with 0.75 % (w/v) agar. For selection using erythromycin, the mixture was overlaid onto BHI agar plates containing sodium citrate (10 mM, pH 7.5) and erythromycin ($1.5 \mu\text{g ml}^{-1}$) and incubated for 2 h at 37 °C. The plates were then overlaid with 3 ml BHI with 0.75 % (w/v) agar and $15 \mu\text{g}$ erythromycin ml^{-1} . Transductants were selected after the plates had been incubated overnight at 37 °C.

Catalase activity during growth of *L. monocytogenes* EGD. Cells from a 1 ml sample of culture were harvested by centrifugation (14000 *g*, 2 min), and incubated in 2.5 mg lysozyme ml^{-1} dissolved in Kpi buffer (0.615 M K_2HPO_4 and 0.385 M KH_2PO_4 in distilled H_2O) at 37 °C for 30 min. Catalase activity was determined by decrease in H_2O_2 concentration (Beers & Sizer, 1952) in Kpi buffer (pH 7.0, 25 °C) using a Shimadzu UV-2401 PC spectrophotometer. Protein concentration was determined colorimetrically (Bio-Rad protein assay reagent), using BSA as standard.

Tn917-LTV3 insertion libraries. Two libraries of Tn917-LTV3 transposon insertional mutants were created using the temperature-sensitive vector pLTV3 as previously described by Camilli *et al.* (1990), with the exception that the cells were grown through three subcultures at 40.5 °C in BHI containing $5 \mu\text{g}$ erythromycin ml^{-1} . The libraries both contained approximately 2×10^{10} c.f.u. ml^{-1} and over 90 % of the cells contained transposon insertions.

Starvation survival mutant selection. A library of transposon mutants was screened as described by Watson *et al.* (1998b), with the exceptions that the glucose-limited CDM agar described above was used and the screen was conducted at 37 °C over 12 d.

Molecular biological methods. All molecular biological methods were performed as described by Sambrook *et al.* (1989). Chromosomal DNA was isolated using the QIAGEN 100/G kit as directed in the manufacturer's manual.

Nucleotide sequence analysis. DNA sequence was obtained directly from purified chromosomal DNA using an ABI BigDye sequencing kit and an ABI 373A DNA sequencer (Applied Biosystems). A 19 bp oligonucleotide (5'-CTCAC-AATAGAGAGATGTCACCGTC-3') complementary to the *lacZ*-proximal end of the transposon was used as sequencing primer. Homology searches were performed using the BLAST search program at the National Centre for Biotechnology Information database (Bethesda, USA).

RESULTS

Establishment of defined starvation survival conditions

To examine the SSR a defined medium for growth of *L. monocytogenes* was determined, based on that described by Trivett & Meyer (1971). Limitation for specific components was carried out as described in Methods. Glucose-limited cultures showed a fall of 90–99.9 % in

Fig. 1. Survival of *L. monocytogenes* EGD at 37 °C. Cells were incubated in glucose-limited CDM (●), amino-acid-limited CDM (○), PBS (■) or distilled H_2O (□). Viability was determined by plating on TSB agar. The initial concentration was approximately 1×10^9 c.f.u. ml^{-1} .

the viable population over the first 2 d. From day 2 onwards, however, there was a significant drop in the rate of fall of viability, with long-term culture viability being retained (Fig. 1). Multiple nutrient limitation elicited by resuspension in PBS or distilled H_2O also resulted in starvation survival (Fig. 1). This was independent of whether the cultures were grown in glucose- or amino-acid-limited medium. In contrast, the viability of cultures incubated in amino-acid-limited CDM at 37 °C declined rapidly, becoming undetectable within 5 d (Fig. 1). A temperature-dependent survival response was seen in glucose-limited cultures, as a higher percentage of the original population was retained after 20 d at 25 °C compared to 37 °C (2.4 % and 0.16 %, respectively; data not shown), although the initial rate of drop in viability was unchanged.

Effect of cell density on starvation survival

Cell density during the SSR may be important as it determines the total potential nutrients available to the population, release of which will require the death of a proportion of the cells. Cells from a 6 h post-exponential-phase glucose-limited culture were harvested by centrifugation and resuspended in water, or the spent culture medium in which they had been grown, at a range of initial cell densities. Upon resuspension in water, survival was seen to be density-dependent as the cells became unculturable within 3 d if resuspended at 2×10^8 c.f.u. ml^{-1} (Fig. 2), but unculturable within 24 h when resuspended at 1×10^7 c.f.u. ml^{-1} or below (data not shown). When resuspended in spent culture supernatant at initial densities of between 7×10^5 and 1×10^9 c.f.u. ml^{-1} , viability in all cultures was retained at approximately

Fig. 2. Effect of cell density on survival of *L. monocytogenes* EGD at 37 °C. Starvation cultures were prepared as described in Methods and harvested as described (Watson *et al.*, 1998a). Cells were resuspended in filter-sterilized culture supernatant to 7×10^5 c.f.u. ml⁻¹ (●) and 1×10^9 c.f.u. ml⁻¹ (○), and in water to 2×10^9 c.f.u. ml⁻¹ (■) and 2×10^8 c.f.u. ml⁻¹ (□). Viability was determined by plating on TSB agar.

5×10^5 – 5×10^6 c.f.u. ml⁻¹ by 20 d (Fig. 2). The survival of cells taken from long-term (7 d) glucose-limited cultures was also density-dependent when incubated in water, all viability being lost within 7 d if resuspended at 2×10^9 c.f.u. ml⁻¹ or below (data not shown). Long-term-starved cells resuspended in spent culture supernatant resulted in cell viability of approximately 5×10^6 c.f.u. ml⁻¹ after 20 d irrespective of initial cell density between 4×10^3 and 5×10^6 c.f.u. ml⁻¹. This implies the presence of a significant level of nutrients within the medium capable of sustaining cellular viability (data not shown).

Effect of starvation on cell morphology

To determine if the SSR results in an altered cellular morphology, as has been observed in other species, cells from 7 d glucose- and amino-acid-limited cultures were observed by electron microscopy in comparison to cells from an exponentially growing culture (data not shown). It was noted that there was large variation of cell length in both exponential-phase and long-term-starved cells. In 7 d glucose-limited cultures, the mean cell length was significantly reduced ($P < 0.01$; Student's *t*-test) with a mean cell length of 1.00 ± 0.26 µm, compared to 1.29 ± 0.29 µm in exponential growth. The mean cell width was also significantly changed ($P < 0.01$; Student's *t*-test), increasing from 0.41 ± 0.03 µm in exponential-phase cells to 0.46 ± 0.05 µm in the glucose-limited cultures. The occurrence of partial division septa was significantly reduced in the long-term glucose-limited cultures, with only 17% seen to possess partial septa compared to 30% of exponential-phase cells. It was noted that large numbers of 'ghost' cells were present in the glucose-limited culture, indicating that

Fig. 3. Effect of penicillin G treatment on survival in glucose-limited CDM. Post-exponential-phase (●, ○) and 7 d glucose-starved (■, □) *L. monocytogenes* EGD cultures were incubated at 37 °C with (●, ■) or without (○, □) penicillin G (20 µg ml⁻¹). Changes in viability were determined by plating on TSB agar. The initial concentration was approximately 1×10^9 c.f.u. ml⁻¹, except for 7 d glucose-starved cultures (5×10^6 c.f.u. ml⁻¹).

cell lysis may be significant. In amino-acid-limited cultures, very few, apparently intact, bacteria were found. The 'cells' observed in these cultures possessed electron-dense fragmented cell walls, with no apparent change in cell dimensions compared to vegetative cells.

Role of cell wall biosynthesis during starvation survival

Penicillin G kills actively growing and dividing cells and so can be used to determine the division status of the population. The addition of penicillin G to 6 h post-exponential-phase cultures increased the rate of loss of viability compared to the control cultures (Fig. 3). After an initial fall of 99% in viability over the first 2 d, similar to that in the controls, viability continued to fall steadily past the point where that of the control cultures stabilized at about 0.2% of the original population (Fig. 3). When penicillin G was added to 7 d glucose-limited cultures, no effect was seen compared to the untreated control (Fig. 3).

Role of protein synthesis during starvation survival

To determine whether continued protein synthesis is required for starvation survival, chloramphenicol (100 µg ml⁻¹) was added at different times during starvation. At 37 °C, protein synthesis inhibition up to 4 h after initiation of glucose limitation resulted in viability becoming undetectable by 10 d, whereas addition of chloramphenicol 8 h post-starvation no longer had an effect on survival for up to 50 d (data not shown).

Fig. 4. Resistance of exponential-phase (●), 6 h post-exponential-phase (○) and 7 d glucose-starved (■) *L. monocytogenes* EGD cells. Cells were resuspended in PBS at an initial concentration of 5×10^6 c.f.u. ml⁻¹. Viability was determined after exposure to pH 3.5 (a), 7.5 mM H₂O₂ (b) or heat stress at 55 °C (c) by plating on TSB agar.

Starvation-induced stress resistance

The effects of exposure to heat (55 °C), low pH (pH 3.5) and oxidative stress (7.5 mM H₂O₂) were tested on cells at different growth phases in glucose-limited cultures (exponential phase, 6 h post-exponential phase and 7-d-starved) (Fig. 4). As the age of the cultures increased, the

Fig. 5. Survival of *L. monocytogenes* EGD (wild-type; ●), DES011 (*sigB*; ▲) and DES012 (*prfA*; ■) in glucose-limited CDM at 37 °C. Viability was determined by plating on TSB agar. The initial concentration was approximately 1×10^9 c.f.u. ml⁻¹.

cells developed a much greater level of resistance to acid (Fig. 4a) and heat stress (Fig. 4c). In fact 7-d-starved cells were over 1000-fold more resistant after exposure to acid or heat (for 60 and 12 min, respectively) compared to exponential-phase cells. H₂O₂ resistance increased during post-exponential phase, and then fell during long-term starvation (Fig. 4b). Resistance to oxidative stress was significantly increased if the cells were retained in the original culture supernatant. Approximately 10% of exponential-phase cells remained viable after 90 min, whilst cells from 6 h post-exponential-phase and 7 d glucose-limited cultures were totally resistant (data not shown).

The role of known regulatory elements in starvation survival and stress resistance

The alternative sigma factor, SigB, is known to be involved in stress resistance during vegetative growth of *L. monocytogenes* (Becker *et al.*, 1998). To determine the role of SigB in the SSR, the *sigB* null mutation from strain LMA2B was transferred into the EGD background by phage transduction to give strain DES011, and verified by Southern blotting (data not shown). During a starvation survival assay in glucose-limited CDM, DES011 showed the same biphasic kinetics as EGD over the first 5 d, but by 20 d the *sigB* mutant had undergone a 10-fold greater drop in viability (Fig. 5). It was also noted that the pH in both EGD and DES011 cultures fell only slightly from a value of 7.0 at the start of incubation to between 6.0 and 6.5 after 20 d. The same changes in cell length and the reduction in the occurrence of partial division septa were observed in DES011 (*sigB*) during the transition into long-term starvation survival as seen in the parental strain EGD (data not shown).

Exponential-phase cells of DES011 were more sensitive to acid treatment than EGD (0.02 and 1% survival after

Fig. 6. Resistance of exponential-phase (●, ▲, ■) and long-term-starved (○, △, □) *L. monocytogenes* EGD (wild-type; ●, ○), DES011 (*sigB*; ▲, △) and DES012 (*prfA*; ■, □) cells. Viability was determined after exposure to pH 3.5 (a) or 7.5 mM H₂O₂ (b). The initial concentration was 5×10^6 c.f.u. ml⁻¹.

25 min, respectively); however, starved cells of both DES011 and EGD were highly acid-resistant (Fig. 6a). The *sigB* mutation had no significant effect on the resistance of exponential-phase and 7-d-glucose-limited cultures to incubation at 55 °C (data not shown). No change in the resistance of DES011 to H₂O₂ was found during post-exponential phase or long-term starvation (Fig. 6b, and data not shown). In exponential phase, however, DES011 exhibited greater H₂O₂ resistance compared to corresponding EGD cells (50 and 1% survival, respectively, after 15 min) (Fig. 6b).

PrfA is a major regulator of virulence determinant production in *L. monocytogenes* and may have a role in other cellular functions (Ripio *et al.*, 1998). The starvation and stress resistance phenotypes of a *prfA* deletion mutant in an EGD background (DES012) were studied. DES012 (*prfA*) shows a consistent reduction in survival during starvation of approximately 10-fold compared to EGD (Fig. 5). It was also noted that DES012 exhibits a 'small colony variant' phenotype on recovery after long-term starvation. However, the same changes in cell length and the reduction in the occurrence

Fig. 7. Catalase activity of *L. monocytogenes* EGD (wild-type; ●), DES011 (*sigB*; ▲) and DES012 (*prfA*; ■). Growth (OD₆₀₀) of EGD (○) is also shown. One unit of catalase activity equals the degradation of 1 µmol H₂O₂ min⁻¹.

of partial division septa occur in DES012 (*prfA*) during the transition into long-term starvation survival as seen in the parental strain EGD (data not shown). The *prfA* deletion had no effect on resistance to heat stress in comparison to the parental strain (data not shown). Exponential-phase cells of DES012 showed 10-fold more resistance to acid treatment after 60 min, whilst starved cells of EGD and DES012 were equally resistant (Fig. 6a). Increased resistance of DES012 to H₂O₂ was also observed. Exponential-phase cells were almost fully resistant to 7.5 mM H₂O₂, with less than a 1 log fall after 50 min (Fig. 6b), whereas EGD became unrecoverable (> 10³-fold loss after 20 min) during the experiment. In cells taken from 7 d glucose-limited cultures, total resistance to H₂O₂ was seen compared to a 3 log reduction in viability in EGD cells.

Catalase activity during growth of *L. monocytogenes* EGD

Catalase is a major factor involved in the resistance of cells to oxidative stress, via degradation of hydrogen peroxide. Catalase activity increased steadily during growth of *L. monocytogenes* EGD to a maximum level in stationary phase [1×10^5 U (mg protein)⁻¹] (Fig. 7). The pattern of catalase activity development in the *sigB* mutant during growth was the same as in EGD; however the levels observed were consistently reduced by up to 64% (8 h, Fig. 7). During mid- to late-exponential phase, catalase activity in the *prfA* mutant increased to over 3.5 times that seen in EGD, reaching a peak after 8 h [1.2×10^5 U (mg protein)⁻¹]. Activity then fell on entry to stationary phase to levels approximately 50% of wild-type levels (Fig. 7). In all three strains, during long-term starvation the cells retain relatively low levels of catalase activity [4.9×10^3 – 1.5×10^4 U (mg protein)⁻¹] (data not shown).

Isolation of starvation survival mutants

To identify further components of the SSR two libraries of Tn917-LTV3 transposon insertional mutants were created using the temperature-sensitive vector pLTV3 (Camilli *et al.*, 1990). These libraries were screened for mutants defective in starvation survival under glucose-limited conditions. Of 9000 clones screened, 21 were found to be starvation survival mutants in the agar plate screen. After phage transduction, four clones were found to exhibit a starvation survival defect on solid media. The defect was linked to the Tn917 insertion (five out of five transductants). There was no recovery of the bacterium upon replica plating (24 h, 37 °C) from glucose-limited CDM agar plates after 12 d incubation at 37 °C.

Analysis of Tn917-LTV3 insertion in starvation survival mutants

Southern hybridization analysis of the mutants revealed that all contained a single transposon (data not shown). DNA sequencing directly from genomic DNA of the mutants revealed that the four clones were two pairs of siblings. The transposon insertion in DES028 and DES029 was found to be located 129 bp downstream of the putative start site of the coding sequence for a homologue of the *B. subtilis* gene *yulB* [D70014; 39% identity (17/43 amino acids)]. DES035 and DES045 were found to have the transposon insertion 69 bp downstream of the putative start site in the coding sequence for a gene (ORF1) whose product had no significant homology to any proteins in the database.

Stress resistance of starvation survival mutants

DES028 and DES045 were chosen as representative of the sibling pairs. When DES028 was incubated at 55 °C, long-term (7 d) glucose-starved cells exhibited a 40-fold reduction in resistance compared to EGD (12 min, data not shown), whilst the same cells were over 30-fold less resistant to 7.5 mM H₂O₂ than EGD (50 min, data not shown). DES045 cells showed no significant change in starvation-associated stress resistance compared to wild-type cells.

DISCUSSION

In the chemically defined medium described here, *L. monocytogenes* develops an SSR in reaction to glucose- or multiple-nutrient limitation, enabling maintenance of viability for prolonged periods. The specificity of the generation of the SSR via carbon- or multiple-nutrient limitation equals that of *Vibrio* sp. and *S. aureus* (Nyström *et al.*, 1992; Watson *et al.*, 1998a).

The development of the SSR is characterized by a rapid fall in viability of 90–99.9%, prior to the long-term maintenance of cell viability. This phenomenon occurs in *S. aureus*, *E. coli* and *Salmonella typhimurium* (Siegele *et al.*, 1993; Spector & Cubitt, 1992; Watson *et al.*, 1998a), and is proposed to be the result of cryptic growth – the maintenance of a small population of cells

via the release of utilizable nutrients from the death and lysis of the majority. Survival kinetics similar to those seen with multiple-nutrient limitation in this report have been found in *L. monocytogenes* Scott A when stored in phosphate buffer (Lou & Yousef, 1996). Establishment of the SSR was found to be cell-density-dependent and so cell death – be it via nutrient limitation and/or osmotic stress – and the subsequent release of nutrients, and possibly also signalling molecules, is required for the maintenance of the long-term viability of the remaining cells. This supports the idea of cryptic growth in these cultures. Further evidence is provided by the fact that spent culture supernatant allows the regrowth of cells resuspended at low cell densities to a consistent concentration.

During prolonged glucose starvation, cells of a number of species undergo a series of morphological changes. In *E. coli*, the RpoS-regulated *bolA* gene is responsible for the starvation-induced reduction in cell length (Aldea *et al.*, 1998; Lange & Hengge-Aronis, 1991). Here, we have shown that *L. monocytogenes* most likely undergoes reductive division during starvation, resulting in shorter cells. Similar reduction in cell dimensions is also witnessed in *S. aureus* and *Micrococcus luteus* (Mukamolova *et al.*, 1995; Watson *et al.*, 1998a); however the molecular basis for this in the Gram-positive bacteria is unknown.

If the population is undergoing cell division then active peptidoglycan biosynthesis must be occurring, rendering them sensitive to the action of penicillin G. In the early stages of starvation, penicillin G has a deleterious effect on viability, which suggests active division is still occurring. However, after 7 d no further effects are seen, and so perhaps by this stage the population has reached stasis with no further cell division. In *L. monocytogenes*, starvation survival is dependent on gross protein synthesis. This correlates with *E. coli* and *S. aureus* (Reeve *et al.*, 1984; Watson *et al.*, 1998a) as the cells synthesize novel proteins in response to starvation conditions. Interestingly, it appears that *L. monocytogenes* no longer synthesizes proteins by 8 h into starvation as the addition of chloramphenicol has no effect on viability. However, the possibility cannot be ruled out that by this stage the organism has a phenotypic tolerance to chloramphenicol.

In other organisms, many of the differentially expressed proteins have been identified, and several have been found to regulate or confer resistance to environmental stresses (Matin, 1991). An increase in cross-resistance to several stresses has been found to occur as cells become starved (Jenkins *et al.*, 1988; Seymour *et al.*, 1996; Watson *et al.*, 1998a). *L. monocytogenes* EGD also shows a starvation-associated increase in resistance to environmental stress. Resistance to H₂O₂ is probably primarily mediated by an extracellular catalase, which is produced throughout growth. Oxidative damage is a major contributor to cell death and ageing in starved cultures (Dukan & Nyström, 1998; Nyström, 1999), and may also occur as a result of treatment with

acid (Clements *et al.*, 1999). The effect of culture supernatant from aged cultures in increasing the apparent resistance of *L. monocytogenes* EGD cells to H_2O_2 is most likely via the protective action of a catalase secreted into the medium, an effect also seen in both *S. aureus* and *B. subtilis* (Leimeister-Wächter *et al.*, 1992; Watson *et al.*, 1998a).

The increase in heat resistance during starvation supports previous findings on the effect of nutrient stress on thermotolerance (Lou & Yousef, 1996). It is known that exposure of *L. monocytogenes* to heat shock increases the expression of at least 14 proteins, including GroEL and DnaK (Hanawa *et al.*, 1995). *B. subtilis* possesses at least four distinct classes of heat-induced proteins regulated by distinct mechanisms: class I (HrcA repressor-dependent), class II (SigB-dependent), class III (CtsR-dependent) and class IV (whose mode of regulation is unknown) (Derré *et al.*, 1999). Studies to date provide evidence that *L. monocytogenes* has the elements of at least three of the classes found in *B. subtilis* (Derré *et al.*, 1999; O'Driscoll *et al.*, 1996; Wiedmann *et al.*, 1998).

The regulation of the SSR is complex and not well understood in Gram-positive bacteria. In *E. coli* and other Gram-negatives, the alternative sigma factor RpoS has a central role; however this component is absent in Gram-positives. In *B. subtilis*, the alternative sigma factor SigB has been recognized as a general stress response regulator (Völker *et al.*, 1999), controlling a regulon of over 50 genes (Antelmann *et al.*, 1997; Bernhardt *et al.*, 1997). SigB has also been found in other Gram-positives, including *S. aureus* and *L. monocytogenes* (Kullik & Giachino, 1997; Wiedmann *et al.*, 1998). Expression of *sigB* in *L. monocytogenes* was found to increase in response to hydrogen peroxide, acid and temperature shock, increased osmolarity and addition of EDTA (Becker *et al.*, 1998), and a *sigB* mutant had reduced acid resistance in stationary phase, but unchanged virulence (Wiedmann *et al.*, 1998). Previous work in our laboratory has shown SigB not to have a major role in starvation survival in *S. aureus* (Chan *et al.*, 1998). In *L. monocytogenes* EGD, however, SigB seems to contribute to starvation survival as the mutant DES011 shows a 10-fold reduction in number of survivors. Low pH is unlikely to be the principal cause of the reduced survival of DES011, though the possibility remains that the cumulative effect of the slightly reduced pH over a prolonged period could be sufficient to produce the phenotype seen. Although SigB is partially responsible for acid resistance during exponential and post-exponential phase, it has no role in the high-level resistance seen in long-term-starved cells. This is equivalent to that seen in *S. aureus* (Chan *et al.*, 1998). Therefore, there are at least two mechanisms of acid resistance in *L. monocytogenes* EGD – one SigB-dependent, and one starvation-associated, SigB-independent. Acid tolerance is important for *L. monocytogenes* as mutants with increased resistance are also more virulent (O'Driscoll *et al.*, 1996). Bacterial acid resistance also has implications in food hygiene, as the

aforementioned acid-tolerant mutants show increased survival potential in a variety of foodstuffs (Gahan *et al.*, 1996). In *L. monocytogenes*, we have found that a SigB mutation results in increased H_2O_2 resistance, but only during exponential phase. This seems to be independent of catalase expression, and only occurs in washed cells. The molecular basis for this phenomenon is unknown. In *S. aureus*, SigB regulates H_2O_2 resistance during both exponential phase and starvation survival (Chan *et al.*, 1998).

Another important regulator of gene expression in *L. monocytogenes* is PrfA. This transcriptional activator regulates the production of the major virulence gene cluster in *L. monocytogenes* in response to environmental signals (temperature, growth phase, iron concentration and pH) in association with an unknown cofactor (Behari & Youngman, 1998; Böckmann *et al.*, 1996; Leimeister-Wächter *et al.*, 1992; Mengaud *et al.*, 1991). PrfA is well known as a homologue of the global regulators Crp and Fnr (Lampidis *et al.*, 1994), and recently the potential role of PrfA in regulating other cellular functions has come to light with the discovery that PrfA negatively regulates ClpC expression (Ripio *et al.*, 1998). ClpC, a member of the Clp ATPase stress protein family, is also known to have a dual role in stress resistance and virulence, with mutants found to be sensitive to iron limitation, increased osmolarity and heat stress, coupled with reduced virulence *in vivo* (Rouquette *et al.*, 1996). Here we have shown that PrfA contributes not only to starvation survival but also to stress resistance. In fact PrfA seems to down-regulate stress resistance mechanisms during exponential growth. This is likely to be due to PrfA being a member of the complex interacting hierarchy of gene regulators which control the physiology of *L. monocytogenes* in response to environmental stimuli.

Catalase activity in *L. monocytogenes* is known to be induced by stresses, namely heat, hydrogen peroxide and salt (Dallmier & Martin, 1998, 1990). Here, catalase activity in *L. monocytogenes* EGD was shown to be at least partially controlled by SigB, and thus the situation correlates with that in *B. subtilis*, where SigB-dependent expression of *katE* forms part of a non-specific oxidative stress response (Engelmann & Hecker, 1996). The fact that a PrfA deletion mutation changes the temporal expression of catalase activity highlights a further example of stress gene regulation and indicates a possible extended role of PrfA into the basic physiology of *L. monocytogenes*. It remains to be seen, however, whether this phenomenon results from the direct action of PrfA.

A number of cellular components have been identified with a role in the initiation and maintenance of starvation survival (Watson *et al.*, 1998b). Here, we have identified two novel loci involved in starvation survival under glucose limitation, to our knowledge the first report of such mutants of *L. monocytogenes*. The insertional mutation in DES028 (DES029) occurred within a homologue of the *yulB* gene from *B. subtilis*, the

gene product of which is a member of the DeoR family of transcriptional regulators. Notably, the majority of these proteins act as activators or repressors of genes involved in sugar utilization (Beck von Bodman *et al.*, 1992). The ability to use alternative carbon sources is of great importance for cell survival when the initial source of glucose is depleted, and mutations within the complex regulatory pathways would be likely to have a deleterious effect on survival. The inability to recycle those carbon sources released during the death and lysis of the majority of cells in the culture may well cause the defect seen in DES028 (DES029). The reduction in resistance to hydrogen peroxide and heat resistance that is also observed during post-exponential phase and long-term starvation may therefore be a result of the mutant being unable to meet the energy requirements needed to resist these stresses. In strain DES035 (DES045), the Tn917 insertion occurred in a gene, the product of which had no significant homology to known proteins. Therefore, a role for this gene in starvation survival cannot yet be determined.

We have shown that *L. monocytogenes* has an SSR that results in increased resistance to environmental stresses, and that a number of genes play a role in the starvation survival of *L. monocytogenes*. This highlights the need to further understand the SSR, as in this state *L. monocytogenes* is highly resistant to environmental stress and so is more difficult to eradicate.

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