The *rpoS*-dependent starvation-stress response locus *stiA* encodes a nitrate reductase (*narZYWV*) required for carbon-starvation-inducible thermotolerance and acid tolerance in *Salmonella typhimurium*

Michael P. Spector,¹ Francisco Garcia del Portillo,^{3†}
Shawn M. D. Bearson,^{2‡} Atif Mahmud,¹ Maureen Magut,¹
B. Brett Finlay,³ Gordon Dougan,⁴ John W. Foster²
and Mark J. Pallen^{4§}

Author for correspondence: Michael P. Spector. Tel: +1 334 380 2710. Fax: +1 334 380 2711.
e-mail: mspector@usamail.usouthal.edu

^{1,2} Department of Biomedical Sciences¹ and Department of Microbiology and Immunology², University of South Alabama, Mobile, AL 36688, USA

³ Biotechnology Laboratory and Departments of Biochemistry and Microbiology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

⁴ Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY, UK

The starvation-stress response (SSR) of *Salmonella typhimurium* includes gene products necessary for starvation avoidance, starvation survival and virulence for this bacterium. Numerous genetic loci induced during carbon-source starvation and required for the long-term-starvation survival of this bacterium have been identified. The SSR not only protects the cell against the adverse effects of long-term starvation but also provides cross-resistance to other environmental stresses, e.g. thermal challenge (55 °C) or acid-pH challenge (pH 2.8). One carbon-starvation-inducible *lac* fusion, designated *stiA* was previously reported to be a σ^S -dependent SSR locus that is phosphate-starvation, nitrogen-starvation and H₂O₂ inducible, positively regulated by (p)ppGpp in a *relA*-dependent manner, and negatively regulated by cAMP:cAMP receptor protein complex and OxyR. We have discovered through sequence analysis and subsequent biochemical analysis that the *stiA::lac* fusion, and a similarly regulated *lac* fusion designated *sti-99*, lie at separate sites within the first gene (*narZ*) of an operon encoding a cryptic nitrate reductase (*narZYWV*) of unknown physiological function. In this study, it was demonstrated that *narZ* was negatively regulated by the global regulator Fnr during anaerobiosis. Interestingly, *narZ*(*YWV*) was required for carbon-starvation-inducible thermotolerance and acid tolerance. In addition, *narZ* expression was induced ~20-fold intracellularly in Madin-Darby canine kidney epithelial cells and ~16-fold in intracellular salts medium, which is believed to mimic the intracellular milieu. Also, a *narZ1* knock-out mutation increased the LD₅₀ ~10-fold for *S. typhimurium* SL1344 delivered orally in the mouse virulence model. Thus, the previously believed cryptic and constitutive *narZYWV* operon is in fact highly regulated by a complex network of environmental-stress signals and global regulatory functions, indicating a central role in the physiology of starved and stressed cells.

Keywords: nitrate reductases, *stiA*, thermotolerance, acid tolerance, starvation-stress response

[†] **Present Address:** Centro de Biología Molecular 'Severo Ochoa', Universidad Autónoma de Madrid, 28049 Madrid, Spain.

[‡] **Present Address:** Department of Microbiology and Molecular Genetics, University of California at Los Angeles, Los Angeles, CA 90095, USA.

[§] **Present Address:** Microbial Pathogenicity Research Group, Department of Medical Microbiology, St. Bartholomew's and the Royal London School of Medicine and Dentistry, London EC1A 7BE, UK.

Abbreviations: CRP, cAMP receptor protein; ISM, intracellular salts medium; LB, Luria-Bertani; MDCK, Madin-Darby canine kidney; MS loPCN, MOPS-buffered salts medium limiting for glucose; MS hiPCN, MOPS-buffered salts medium non-limiting for glucose, phosphate and nitrogen; SP-PCR, single-primer polymerase chain reaction; SSR, starvation-stress response; NR, nitrate reductase.

INTRODUCTION

The microenvironments that bacteria, such as the serovars of *Salmonella enterica* (e.g. *S. enterica* serovar Typhimurium, referred to here as *S. typhimurium*), encounter as they cycle from their natural animal or human hosts to various aquatic and terrestrial microcosms are frequently limiting in the bioavailability of nutrients and fraught with a variety of potentially lethal stresses (Koch, 1971; Harder & Dijkhuizen, 1983; Roszak & Colwell, 1987; Morita, 1988; Moriarty & Bell, 1993; Foster & Spector, 1995). Therefore, *Salmonella* must be able to sense, respond to and adapt to changing environments in order to survive extensive periods of nutrient starvation and/or other stresses while retaining their pathogenic capabilities (Brown & Williams, 1985; Babior, 1992; Foster & Spector, 1995). Thus, the response and survival strategies of these bacteria may have important consequences for the epidemiology and virulence of salmonellosis.

The common stress of starvation for essential nutrients, especially an energy-yielding carbon (C) source, leads to a myriad of changes in cellular metabolism and patterns of gene expression in the bacterium referred to as the starvation-stress response (SSR) (Foster & Spector, 1986; Spector *et al.*, 1986, 1988; Spector, 1990, 1998; Spector & Foster, 1993). The function of the SSR is to allow the bacteria to survive periods of long-term starvation (Spector & Cubitt, 1992; Spector & Foster, 1993; O'Neal *et al.*, 1994). In addition, induction of the SSR, particularly by C starvation, increases bacterial resistance to a number of other environmental stresses, such as the presence of H₂O₂ or antimicrobial peptides, and extremes in temperature, pH and osmolarity (Jenkins *et al.*, 1988; Matin, 1991; Lange & Hengge-Aronis, 1991; McCann *et al.*, 1991; Fang *et al.*, 1992; Seymour *et al.*, 1996; McLeod & Spector, 1996; M. P. Spector, unpublished).

Over the past several years, we have created a library of starvation-inducible loci using MudJ-directed operon-fusion techniques (Castilho *et al.*, 1984; Spector *et al.*, 1988, 1999; reviewed by Spector, 1998). Two of the fusions (designated *stiA* and *sti-99*) identified from this library are induced during C, phosphate (P) and nitrogen (N) starvation. The loci defined by the *stiA* and *sti-99* insertion mutations are dependent upon the starvation-/stress-specific sigma factor encoded by the *rpoS* gene, σ^S or σ^{38} (Mulvey & Loewen, 1989; Tanaka *et al.*, 1993; for reviews see Loewen & Hengge-Aronis, 1994; Hengge-Aronis, 1993, 1996; Spector, 1998). Both insertion mutations reduced the long-term-starvation survival of *S. typhimurium* some 50- to 100-fold (Spector & Cubitt, 1992; O'Neal *et al.*, 1994; M. P. Spector, unpublished) and thus represent core SSR loci. In addition, both the *stiA* and *sti-99::lac* fusions are negatively regulated by the cAMP:cAMP receptor protein (CRP) complex and positively regulated by guanosine tetraphosphate (ppGpp) in a *relA*-dependent manner (Spector & Cubitt, 1992; M. P. Spector, unpublished; for review see Cashel *et al.*, 1996).

The *stiA* and *sti-99* products are also needed to generate H₂O₂-inducible H₂O₂ resistance (Seymour *et al.*, 1996; M. P. Spector, unpublished). The H₂O₂-inducible H₂O₂ resistance pathway is controlled by the products of both the *oxyR* and *rpoS* genes (Seymour *et al.*, 1996; Storz *et al.*, 1990; for review see Storz & Altuvia, 1994). Interestingly, the *stiA* locus is repressed by the OxyR protein (reduced form) in the absence of H₂O₂ in exponential-phase cells. In the presence of H₂O₂, *stiA* becomes derepressed, presumably as a result of oxidation of the OxyR protein, so that OxyR (oxidized form) no longer functions as a repressor of *stiA* in exponential-phase cells. However, the H₂O₂-induced levels of *stiA* are only about one-third of the level observed in C-starved cells, probably resulting from repression mediated by the cAMP:CRP complex in exponential-phase cells (Spector & Cubitt, 1992; Seymour *et al.*, 1996). Thus, the *stiA* and *sti-99::lac* fusions are positively regulated by σ^S and (p)ppGpp, and negatively regulated by cAMP:CRP and OxyR.

We report here that the MudJ (*lac*) fusions defining the *stiA* and *sti-99* loci are located at different sites in the same gene. Furthermore, this gene is orthologous to *Escherichia coli narZ*, the first gene of the *narZYWV* operon which encodes a nitrate reductase (NR) activity previously thought to be cryptic and constitutive (NR-Z) (Barrett & Riggs, 1982; Blasco *et al.*, 1990; Bonnefoy & DeMoss, 1994). As previously reported for both *E. coli* and *Salmonella*, NR-Z, unlike the *narGHJI*-encoded NR-A, is not synthesized during anaerobiosis nor is it responsive to exogenously added nitrate during anaerobiosis or aerobiosis (Barrett & Riggs, 1982; Bonnefoy & DeMoss, 1994; reviewed by Gennis & Stewart, 1996). In fact, *narZYWV* was repressed during anaerobic growth conditions by the *fnr* gene product, Fnr. We also report that NR-Z was required for C-starvation-inducible thermotolerance and acid tolerance and was significantly induced within vacuoles of MDCK epithelial cells (Garcia del Portillo *et al.*, 1992) in culture as well as in intracellular salts medium (ISM) (Wilson *et al.*, 1997). Furthermore, a knock-out mutation of *narZ* increased the LD₅₀ of wild-type cells some 10-fold following oral inoculation in the mouse virulence model for *Salmonella*.

METHODS

Bacterial strains, phage and transductions. The strains used in this study, unless otherwise indicated, were all derivatives of the virulent *Salmonella enterica* serovar Typhimurium (referred to here as *Salmonella typhimurium*) strain SL1344 and are listed in Table 1. Transductions were performed using a high transducing derivative of *S. typhimurium* bacteriophage P22, P22 HT 105/1 *int* (HT phage) (Chan *et al.*, 1972). In all cases, transductants were determined to be non-lysogens for phage P22 by growth on Green indicator plates (Davis *et al.*, 1980) and sensitive to the H5 derivative of P22 (Maloy, 1990).

Culture media, supplements and antibiotics used. The minimal medium used in this study was a modified MOPS-buffered salts (MS)-based medium (Neidhardt *et al.*, 1974; described in detail by Spector *et al.*, 1988; Spector & Cubitt, 1992). MS medium nonlimiting in glucose, phosphate and

Table 1. *Salmonella enterica* sv. Typhimurium strains used in this study

Strain	Genotype (pertinent phenotype)*	Source/reference
SL1344	<i>bisG46</i> (virulent <i>Salmonella typhimurium</i>)	Hoiseth & Stocker (1981)
ST63	SL1344 <i>narZ2</i> (<i>sti-99</i>)::MudJ (<i>lac Kan^R</i>)	This study
ST66	SL1344 <i>narZ1</i> (<i>stiA1</i>)::MudJ (<i>lac Kan^R</i>)	Spector <i>et al.</i> (1988); O'Neal <i>et al.</i> (1994)
SMS438	SL1344 <i>rpoS</i> :: Ω -Amp ^R	Fang <i>et al.</i> (1992); O'Neal <i>et al.</i> (1994)
SMS575	ST66 <i>fnr</i> ::Tn10 (Tet ^R)	This study; J. Foster†
SMS643	SL1344 <i>narGHIJ1715</i> ::MudJ (<i>lac Kan^R</i>)	This study; V. Stewart‡

* Kan^R, kanamycin resistance; Amp^R, ampicillin resistance; Tet^R, tetracycline resistance.

† John Foster, University of South Alabama, USA.

‡ Valley Stewart, University of California, Davis, USA.

nitrogen (MS hiPCN) was used to generate exponential-phase cells while MS medium limiting in glucose (0.03 % w/v; MS loC) was used to generate C-starved cells. The rich medium used was Luria-Bertani (LB) agar and broth (Davis *et al.*, 1980; Difco). ISM was obtained from Dr Paul Gulig, University of Florida College of Medicine (Wilson *et al.*, 1997).

Histidine was added, as needed, to a final concentration of 0.2 mM. Kanamycin (Kan) and chloramphenicol (Cam) were each used, as needed, at a final concentration of 100 µg ml⁻¹. Tetracycline (Tet) was used at the final concentration of 20 µg ml⁻¹ in rich medium and 10 µg ml⁻¹ in minimal medium. Ampicillin (Amp) was used at a final concentration of 30 µg ml⁻¹.

Analysis and DNA sequencing of *S. typhimurium* DNA flanking the *stiA* and *sti-99* MudJ insertion sites. DNA adjacent to the *stiA*::MudJ insertion site was cloned by digesting chromosomal DNA isolated from strain ST66 with *SalI*, using protocols supplied by the manufacturer (Promega). *SalI* was chosen because there is a single site within MudJ, about 2.5 kb from the left end of MudJ and just upstream from the Kan^R gene, so that *SalI* restriction fragments of 2.5 kb or greater would contain the Kan^R gene and remaining MudJ DNA plus any chromosomal DNA adjacent to the insertion site. *SalI* fragments were separated in a TBE 0.7 % agarose gel. Restriction fragments between 2.5 and 8 kb were purified by cutting out the corresponding region from the gel, freeze-thawing and mashing the gel slice with a sterile pestle, adding 100 µl TE (10 mM Tris, 1 mM EDTA) buffer and subjecting the mixture to filtration through a ULTRAFREE-MC filter unit (Millipore). The filtrate was collected, ethanol precipitated and resuspended in 50 µl sterile distilled water (target DNA). Target DNA was then mixed with *SalI*-digested pACYC184, ligated with T4 ligase and electrotransformed into electrocompetent recipient cells, selecting for Kan^R (target DNA) and Cam^R (vector DNA). Kan^R Cam^R transformants were picked, and their plasmids were isolated using Wizard Mini-prep columns (Promega), digested with *SalI* and analysed by TBE 0.7 % agarose gel electrophoresis. The plasmid carrying the desired fragment, pSS1, was then used for sequencing of the target DNA via dideoxynucleotide sequencing using protocols supplied by the manufacturer (Promega).

The insertion sites for both the *stiA*::*lac* and *sti-99*::*lac* were also analysed using an adaptation of the single-primer PCR (SP-PCR) method of Parks *et al.* (1991), which is described in detail by Spector *et al.* (1999). SP-PCR was performed on cell lysates from ST63 and ST66 and the wild-type parent SL1344.

Briefly, this method relies on the use of a single PCR primer designed to prime DNA synthesis out of the left end of MudJ (MudL; GenBank accession no. M10190) into adjacent chromosomal DNA. However, exponential amplification of the MudJ/chromosome junction site only occurs if the MudL primer also binds to a stretch of partially complementary sequence in the region of unknown sequence on the opposite strand. Fortunately, this occurs at a sufficiently high frequency so as to be useful if one or more of the PCR cycles includes a low annealing temperature. If successful, this primer should yield a product possessing a 34 bp sequence between the primer-binding site and the end of the MudL sequence at one of its ends. Products not containing this 34 bp region were considered artifacts of the procedure and not studied further.

PCR products were first analysed and then ligated to the pGEM-T plasmid prior to being transformed into hyper-competent HB101 cells (supplied with the vector) according to the manufacturer's instructions (Promega). White colonies on LB Amp IPTG X-Gal plates (which contain vector and insert) were subcultured, and their plasmids were isolated and analysed via PCR using forward and reverse vector-specific primers (Promega). Five microlitre aliquots of the completed reactions were run on a TBE 1 % agarose gel to determine the presence and size of any insert. Amplified inserts were then PEG precipitated (Rosenthal *et al.*, 1993) and sequenced by *Taq* cycle sequencing using forward and/or reverse primers with subsequent analysis on an ABI 377 automated sequencer (Applied Biosystems).

Growth and starvation conditions. The desired strains were grown overnight in MS hiPCN, with antibiotic as needed, at 37 °C with shaking. Overnight cultures were then diluted 1:100 into 4 ml fresh MS hiPCN with shaking in a 20 mm test tube (to generate exponential-phase aerobically grown cells) or static (overlaid with sterile mineral oil) in a 13 mm test tube (to generate exponential-phase anaerobically grown cells) with or without 50 mM potassium nitrate, or 4 ml fresh MS loC with shaking in a 20 mm test tube, and incubated at 37 °C. Growth was monitored by measuring A₆₀₀. Cells were grown in MS hiPCN to A₆₀₀ of 0.3–0.4 (exponential phase) with or without nitrate and assayed for nitrate reductase activity and/or β -galactosidase activity. Three-hour C-starved cells were obtained by growing the cells in MS loC broth until growth stopped as a result of exhaustion of glucose and continuing to starve the cells for a total of 3 h, at which point cells were assayed for nitrate reductase and/or β -galactosidase activity.

Enzyme assays. NR activity was measured at desired time intervals. Depending on whether high or low activity was

expected, 0.5 ml or 1.5 ml, respectively, of culture was removed, centrifuged and the supernatant discarded. The cells were then washed twice in 1 ml MS buffer (to remove any NO₂ present). The washed cells were resuspended in 1 ml MS buffer plus 5 mM KNO₃ and incubated for 30 min at 37 °C. The suspension was then centrifuged and 0.5 ml supernatant was mixed with 0.5 ml Greiss reagent (Sigma) and allowed to develop at room temperature for 15 min. The A₅₄₀ was then measured and recorded. Nanomoles of NO₂ produced were determined from a standard curve for known NO₂ concentrations. NR activity was measured as nmol NO₂ formed ÷ (A₆₀₀ × 15 min × 0.5 or 1.5 ml cells); where A₆₀₀ is A₆₀₀ of culture at the point of assay. An NR unit equals 1 nmol NO₂ formed min⁻¹ (A₆₀₀ × vol. cells)⁻¹. β-Galactosidase activity was measured by the method of Miller and is expressed in Miller units (Miller, 1972; 1992).

Thermotolerance and acid-tolerance protocols. To test for C-starvation-inducible resistance mechanisms, cells were grown as described above. At appropriate time intervals, aliquots of aerobically grown exponential-phase, 5 h C-starved and 24 h C-starved cells were diluted 1:100 in MS buffer at 55 °C (for thermotolerance) or MS buffer at pH 2.8 (for acid tolerance). To determine survival during thermal challenge, aliquots were removed every 4 min from time zero up to a maximal challenge period of 20 min and serially diluted prior to plating onto LB medium plus antibiotic as needed. For acid-challenge-survival determination, aliquots were removed at time zero, 10 min, 30 min and 45 min, and serially diluted prior to plating onto LB medium, plus antibiotic as needed. The percentage survival was calculated as the c.f.u. ml⁻¹ at each time point divided by the c.f.u. ml⁻¹ at time zero (maximum viability), which was typically 3–5 × 10⁶ c.f.u. ml⁻¹.

Assay for expression in MDCK cells. Infection and assay for intracellular β-galactosidase expression was carried out as described previously (Finlay & Falkow, 1989; Garcia del Portillo *et al.*, 1992; Spector *et al.*, 1999). Briefly, Madin-Darby canine kidney (MDCK) epithelial cells (ATCC CCL-34) were grown to confluency in minimum Eagle's medium (MEM) containing 5% foetal bovine serum (FBS) in 96-well plates (~10⁵ cells per well). Monolayers were infected with about 10⁶ bacteria, previously grown overnight in LB broth at 37 °C without shaking. Bacterial infection was performed for 2 h at 37 °C. Infected epithelial cells were washed three times with PBS, pH 7.4, to remove non-adherent bacteria. Infected monolayers were then incubated in the presence of fresh MEM plus 5% FBS medium containing 100 µg gentamicin ml⁻¹, to kill any adherent non-internalized bacteria associated with the cell monolayer. Incubation with gentamicin was carried out for the period of 2–4 h post-infection. The gentamicin concentration was then reduced to 10 µg ml⁻¹ and the monolayers were incubated for an additional 2 h (4–6 h post-infection). At 6 h post-infection, the MDCK monolayers were lysed with 1% (v/v) Triton X-100 in PBS (pH 7.4) to release the intracellular bacteria. Viable cell counts were determined for the intracellular bacteria.

A cell lysate was also prepared to measure β-galactosidase activity in intracellular bacteria. Infected epithelial cells were lysed with 0.1% (w/v) SDS/chloroform and β-galactosidase activity was measured using the fluorescent substrate fluorescein di-(β-D-thiogalactopyranoside) as described previously (Garcia del Portillo *et al.*, 1992).

In addition, β-galactosidase activity was measured in parallel-run uninfected epithelial cells and subtracted as the background. Extracellular (non-internalized) bacteria after 2 h incubation with MDCK monolayers were also collected,

viable cell counts determined and β-galactosidase activity measured for comparison with intracellular expression. Intracellular induction was expressed as a ratio between β-galactosidase activity in internalized and extracellular bacteria.

Virulence assays. Cultures of the *S. typhimurium* strains to be tested were grown overnight in L broth at 37 °C without shaking. Cultures were pelleted by centrifugation at 2700 g in a bench-top centrifuge. The cells were then resuspended in PBS. The OD₆₅₀ was adjusted to 0.93, which equates to a cell density of approximately 5 × 10⁸ cells ml⁻¹. For the LD₅₀ determinations, serial dilutions were then made in PBS. Bacterial suspensions were administered intra-gastrically to 6–8-week-old female BALB/c mice by oral gavage in a volume of 200 µl. Mice were observed daily throughout the experiments.

RESULTS

Sequence analysis of chromosomal DNA adjacent to the MudJ insertion sites of *stiA* and *sti-99*

Two independently isolated strains carrying MudJ (*lac*) insertions exhibiting C-starvation-induction were identified from a C-starvation-inducible *lac*-fusion library (Spector *et al.*, 1988, 1999). One of these was designated *stiA* and was previously characterized (Spector *et al.*, 1986, 1988; Spector, 1990; Spector & Cubitt, 1992; O'Neal *et al.*, 1994; Seymour *et al.*, 1996). The other was designated *sti-99*. Both *lac* fusions were found to be induced during C, N and P starvation in a σ^S-dependent manner (O'Neal *et al.*, 1994; M. P. Spector, unpublished; reviewed by Loewen & Hengge-Aronis, 1994). In addition, both are induced when intracellular NAD levels fall to growth-inhibiting concentrations (Spector *et al.*, 1988; M. P. Spector, unpublished). Both are under positive control by *relA* (i.e. ppGpp) and negative control by cAMP:CRP complex (Spector & Cubitt, 1992; M. P. Spector, unpublished). Both were also required for long-term starvation survival of *S. typhimurium* (Spector & Cubitt, 1992; O'Neal *et al.*, 1994; M. P. Spector, unpublished).

Analysis of chromosomal DNA adjacent to the *stiA* and *sti-99*::MudJ (*lac*) insertions was performed using one or both of two protocols. Originally, the kanamycin-resistance gene of the *stiA*::MudJ (*lac*) insertion and downstream chromosomal DNA was cloned utilizing the presence of a unique *SalI* site upstream of the kanamycin-resistance gene in the MudJ vector. A 5 kb *SalI* fragment was cloned into pACYC184, to create pSS1, and used as a template for DNA sequence analysis. Chromosomal DNA adjacent to the *sti-99*::MudJ (*lac*) insertion, as well as the *stiA*::MudJ (*lac*) insertion, was amplified using a SP-PCR protocol (Spector *et al.*, 1999) and the DNA sequence was determined. Results of the DNA sequence analysis obtained from the latter protocol is presented in Fig. 1. The sequence data obtained from the pSS1 *SalI* insert agreed with the sequence obtained from the SP-PCR-amplified fragment presented for *stiA* in Fig. 1. BLAST(P) searches of the sequences obtained revealed that both were located at different sites within the *S. typhimurium* homologue of the *E. coli*

Fig. 1. Comparison of the DNA and deduced amino acid sequences adjacent to the MudJ insertion sites designated *stiA* and *sti-99* in *S. typhimurium* with the *E. coli narZ* DNA and deduced amino acid sequences (GenBank entry X17110). (a) Schematic representation of the *E. coli narZYWV* operon organization and deduced size of each protein. (b) Direct comparison of the primary sequence of the *E. coli narZ* deduced amino acids 598–693 and amino acids 1001–1035 with the deduced amino acid sequences adjacent to the *sti-99::MudJ* insertion site (ST63 *sti-99* deduced) and *stiA::MudJ* insertion site (ST66 *stiA* deduced), respectively. Asterisks (*) indicate shared identical amino acid residues; differences in deduced amino acid residues are indicated for the *Salmonella* sequence. (c) Table showing the number of nucleotides sequenced adjacent to each insertion and the corresponding *E. coli narZ* DNA and deduced amino acid sequence to which it was compared along with the percentage identity they share.

Table 2. Nitrate reductase activity in wild-type, *narGHJ* knockout and *narZYWV* knockout mutant strains of *S. typhimurium* grown under various inducing and non-inducing conditions

Values shown are means $\pm$ SEM of at least three separate experiments.

Strain	Pertinent genotype	NR activity (NR units)		
		Aerobiosis, no nitrate, exponential phase	Anaerobiosis, 50 mM nitrate, exponential phase	Aerobiosis, no nitrate, 3h C starved
SL1344	<i>narZYWV</i> ⁺ <i>narGHJ</i> ⁺	0.24 $\pm$ 0.01	3.44 $\pm$ 0.33	1.21 $\pm$ 0.21
SMS643	<i>narZYWV</i> ⁺ <i>narGHJ1715::MudJ</i>	0.23 $\pm$ 0.02	0.22 $\pm$ 0.01	1.15 $\pm$ 0.15
ST66	<i>narZ1::MudJ</i> <i>narGHJ</i> ⁺	0.24 $\pm$ 0.03	2.82 $\pm$ 0.55	0.21 $\pm$ 0.11

narZ gene sequence (GenBank entry X17110). The MudJ (*lac*) insertion designated *sti-99* was found to be proximal (deduced amino acid 597 of 1246) to the deduced translational start site, followed by the more distal MudJ (*lac*) insertion designated *stiA* (deduced amino acid 1000 of 1246). As a result, the *stiA* and *sti-99* insertions were renamed *narZ1* and *narZ2*, respectively, to reflect these findings.

Phenotypic evidence to support the identification of the *stiA* locus as *narZ*(*YWV*)

To support our DNA sequence analyses indicating that the *stiA/sti-99* locus was identical to the *narZ* gene, phenotypic characterization was carried out. The *narZ*

gene is the first gene of the *narZYWV* operon which encodes a previously described cryptic and constitutive NR activity, NR-Z. Unlike the major NR of *E. coli* and *Salmonella*, NR-A, encoded by the *narGHJ* operon (reviewed by Gennis & Stewart, 1996), NR-Z production is not anaerobically induced and is not nitrate responsive. The data presented in Table 2 illustrate that there are, at least, two detectable NR activities in *S. typhimurium* SL1344, one anaerobiosis and nitrate responsive, and the other C-starvation inducible. As shown in Table 2, the anaerobiosis- and nitrate-responsive NR activity was eliminated by the *narG*(*HJI*) knock-out insertion mutation but not by *narZ1* knock-out insertion mutation (data are shown for the *narZ1* insertion mutation but similar data were obtained for

Table 3. Role of Fnr in *narZYWV* operon expression in *S. typhimurium*

Values are means $\pm$ SEM of at least three separate experiments.

Strain	Pertinent genotype	β -Galactosidase activity (Miller units)		
		Aerobiosis, exponential phase	Anaerobiosis, exponential phase	Aerobiosis, 3h C starved
ST66	<i>narZ1::lac</i>	9.88 $\pm$ 1.65	0.18 $\pm$ 0.25	97.59 $\pm$ 37.03
SMS575	<i>narZ1::lac fnr::Tn10</i>	25.25 $\pm$ 3.85	13.13 $\pm$ 1.33	102.90 $\pm$ 21.32

Fig. 2. Role of NR-Z in C-starvation-inducible thermotolerance. Wild-type (●) and *narZ1::lac* mutant (▲) cells were grown aerobically to exponential-phase (a) and were C-starved for 5 h (b) and 24 h (c) prior to being challenged at 55 °C for a maximum of 20 min. Values represent means $\pm$ SEM of at least three separate experiments.

the *narZ2* knockout-insertion mutation) while the reverse was found for the C-starvation-inducible NR activity. Since the *narZ1(stiA)::lac* fusion and mutation was previously characterized, it was employed for the analyses performed in this study (unless otherwise indicated).

The results presented in Table 3 further support that the *stiA/sti-99* insertions are in the *Salmonella narZ(YWV)* homologue. Previous studies in *E. coli* found that NR-Z activity is repressed anaerobically by the *fnr* gene product, Fnr (Iobbi-Nivol *et al.*, 1990). We found that an *fnr* knock-out mutation resulted in the derepression of *narZ1::lac* expression in anaerobically growing cells (compare anaerobiosis, exponential-phase ST66 and SMS575 in Table 3) to about the level observed in aerobically growing wild-type cells (compare anaerobiosis, exponential-phase SMS575 with aerobiosis, exponential-phase ST66 in Table 3). The *fnr* mutation also led to an approximately 2.5-fold increase in *narZ1::lac* basal expression in aerobically growing cells (compare aerobiosis, exponential-phase ST66 and SMS575 in Table 3). Thus, the *S. typhimurium narZ(YWV)* operon is repressed by Fnr during anaerobiosis as well as during aerobiosis. However, the *fnr* mutation did not affect the level to which *narZ(YWV)* was induced

during C starvation (compare aerobiosis, 3 h C-starved ST66 and SMS575 in Table 3).

Role of the *narZ(YWV)* operon in C-starvation-inducible thermotolerance

We previously reported that the *stiA* locus was required for long-term-starvation survival and H₂O₂-inducible H₂O₂ resistance but it did not affect C-starvation-inducible H₂O₂ resistance (Spector & Cubitt, 1992; Seymour *et al.*, 1996). We wanted to test whether it was also required for heat-shock and C-starvation-inducible thermotolerance. Results from subsequent experiments showed that the *narZ(YWV)* operon is required for thermotolerance generated in both 5 h and 24 h C-starved cells, compared to similarly starved wild-type cells (Fig. 2). The 5 h C-starved *narZ* mutant survived to about the same extent as exponential-phase wild-type or *narZ* mutant cells (compare Fig. 2a and b) and all were clearly more heat sensitive than 5 h C-starved wild-type cells. Although 24 h C-starved *narZ* mutant cells survived thermal challenge better than 5 h C-starved *narZ* mutant cells, they were still significantly more heat sensitive than 24 h C-starved wild-type cells (Fig. 2c). Similar results were found for an *rpoS* mutant, with the

Fig. 3. Role of NR-Z in C-starvation-inducible acid tolerance. Wild-type (●) and *narZ1::lac* mutant (▲) cells were grown aerobically to exponential phase (a) and were C starved for 5 h (b) and 24 h (c) prior to being challenged at pH 2.8 for a maximum of 45 min. Values represent means $\pm$ SEM of at least three separate experiments.

exception that there was no significant difference between exponential-phase, 5 h C-starved or 24 h C-starved cells in terms of thermotolerance generated (data not shown).

Interestingly, the *narZ* knock-out mutation had no effect on heat-shock-inducible thermotolerance in exponential-phase cells compared to the wild-type strain (data not shown). In contrast, exponential-phase *rpoS* mutant cells failed to generate a heat-shock-inducible thermotolerance response (data not shown). Thus, NR-Z appears to be required for C-starvation-inducible thermotolerance but not adaptive heat-shock-inducible thermotolerance.

Role of the *narZ*(YWV) operon in C-starvation-inducible acid tolerance

We also wanted to determine whether *narZ*(YWV) is required for C-starvation-inducible acid tolerance. As shown in Fig. 3, 5 h C-starved wild-type cells were more tolerant than 24 h C-starved wild-type cells to a 45 min pH 2.8 challenge but both were significantly more acid tolerant than exponential-phase cells, which were undetectable after only 10 min at pH 2.8. Interestingly, introduction of a *narZ1* knock-out mutation into wild-type cells reduced acid tolerance generated in 5 h C-starved cells by some 40–50-fold to about the level observed in 24 h C-starved cells (Fig. 3b, c). However, no significant difference was observed in the level of acid tolerance generated in 24 h C-starved *narZ* mutant cells compared to 24 h C-starved wild-type cells (Fig. 3c). In comparison, an *rpoS* mutation had a much more dramatic effect on acid tolerance generated in both 5 h C-starved and 24 h C-starved cells (data not shown) in agreement with similar findings reported by Lee *et al.* (1994). Thus, the *narZYWV* operon is required for acid tolerance generated in 5 h C-starved cells but not 24 h C-starved cells. The reason why the loss of NR-Z reduces acid tolerance in 5 h C-starved cells but not 24 h C-starved cells is unclear at this time. It may indicate that

NR-Z interacts with (an)other function(s), or other acid-tolerance factors are expressed, in 5 h C-starved cells which are then lost as cells continue to starve. It is possible that NR-Z may also disappear over this time period but previous studies show that *narZ1(stiA)::lac* is still transcribed at a relatively high level in 24 h C-starved cells (Spector *et al.*, 1988).

The *narZ* knock-out mutation did not have an effect on acid-shock-inducible acid tolerance generated in exponential-phase cells compared to the wild-type strain (data not shown). Similar to the heat-shock scenario, exponential-phase *rpoS* mutant cells failed to generate an acid-shock-inducible acid tolerance (Lee *et al.*, 1994). Thus, NR-Z appears to be required for C-starvation-inducible acid tolerance but not adaptive acid-shock-inducible acid tolerance.

Expression of the *narZ*(YWV) operon within cultured MDCK epithelial cells and during growth in ISM

S. typhimurium is a facultative intracellular pathogen (Finlay & Falkow, 1989); thus, it is useful to know whether a specific function is expressed in the host environment. This information can provide important insights into the intracellular environment as well as potential roles in pathogenesis (Mahan *et al.*, 1995; Valdivia & Falkow, 1997). To determine whether *narZYWV* is expressed intracellularly within MDCK cells, strains ST66 and ST63 were allowed to infect MDCK epithelial cells. At 6 h post-infection, intracellular bacteria were collected and β -galactosidase activity was measured and compared to the activity in extracellular bacteria. Results from such experiments indicate that *narZYWV* was induced 19.58 ± 5.72 -fold (mean $\pm$ SEM, $n = 4$) within MDCK epithelial cells. Furthermore, the *narZ1::lac* fusion was found to be induced 15.8 ± 0.82 -fold (mean $\pm$ SEM, $n = 3$) during exponential-phase growth in ISM (Wilson *et al.*, 1997) compared to LB broth. ISM was designed to mimic the intracellular milieu of cells and so induction in ISM supports the

findings that NR-Z is expressed within MDCK epithelial cells. The induction ratios observed under both conditions were comparable to the ~10-fold induction seen in 3 h C-starved cells compared to exponentially growing cells but much less than the almost 55-fold induction seen with exponential-phase cells grown aerobically versus anaerobically (Table 3). These results support a model that the intracellular environment seen by *Salmonella* inside MDCK epithelial cells, and perhaps other epithelial cells, generates similar (or cross-reacting) signals to those signals known to induce *narZYWV* expression.

Effect of the *narZYWV* knock-out mutation on the oral LD₅₀ of SL1344 in the mouse virulence model

Since *narZYWV* was induced intracellularly, we tested to see whether the *narZ1* mutation had an effect on *S. typhimurium* virulence in the mouse virulence model. For this, SL1344 and its *narZ1* derivative (ST66) were each given by oral gavage to separate groups of five BALB/c mice to determine the relative LD₅₀ values. Mice were monitored for 28 d. The LD₅₀ values were calculated to be approximately 10^{4.5} for SL1344 and 10^{5.6} for ST66 (a little more than 10-fold higher). Thus, the *narZ* knock-out mutant exhibited a higher LD₅₀ compared to SL1344 but did not completely attenuate virulence when delivered orally in the mouse virulence model.

DISCUSSION

In this study, we report that two independently isolated MudJ (*lac*) insertions, designated *stiA* and *sti-99*, identified from our library of C-starvation-inducible MudJ (*lac*) transcriptional fusions are located at different sites within the same gene. Sequence analysis of chromosomal DNA adjacent to each insertion site showed that both lie within the *S. typhimurium* homologue of the *E. coli narZ* gene (GenBank entry X17110) exhibiting on average >85% identity at the amino acid level (Fig. 1). As mentioned above, the *narZ* gene is the first gene of the *narZYWV* operon encoding NR-Z, previously believed to be both cryptic and constitutive. The *narZ*(YWV) locus of both *E. coli* and *Salmonella* exhibits a high degree of homology at both the DNA and amino acid level to the major anaerobiosis- and nitrate-responsive NR, NR-A, encoded by the *narGHJI* operon. NR-Z was proposed to be constitutive since it was not induced under anaerobic conditions in the presence of nitrate (Iobbi-Nivol *et al.*, 1990; reviewed by Gennis & Stewart, 1996). Results from this study confirm that the *narZYWV* encoded NR-Z is not anaerobiosis- or nitrate-responsive but is induced during C starvation (Table 2). In addition, we present data that *Salmonella narZYWV* expression is repressed under anaerobiosis by Fnr, supporting a previous finding that *narZYWV* in *E. coli* is repressed anaerobically by Fnr (Iobbi-Nivol *et al.*, 1990; Table 3). Thus, we clearly demonstrate that the previously characterized *stiA* locus is identical to the *narZYWV* operon.

The finding that *stiA* is *narZYWV* has some interesting and important implications. The regulation of the *stiA* locus has been studied extensively (Spector *et al.*, 1986, 1988; Spector, 1990; Spector & Cubitt, 1992; O'Neal *et al.*, 1994; Seymour *et al.*, 1996; reviewed by Spector & Foster, 1993; Spector, 1998). Results from this and previous studies show that *narZ*(YWV) is: (a) induced during P and N starvation as well as C starvation, (b) σ^S -dependent, (c) positively regulated by (p)ppGpp in a *relA*-dependent manner, (d) repressed by cAMP:CRP in exponential-phase cells, (e) H₂O₂ inducible and repressed by OxyR (reduced-form) in exponential-phase cells in the absence of exogenous H₂O₂ and (f) repressed by Fnr under anaerobiosis as well as aerobiosis (Table 3). Therefore, NR-Z is anything but constitutive and, in fact, exhibits very complex regulation, foretelling its role in multiple stress responses.

Since NR-Z was not anaerobiosis or nitrate responsive it was originally proposed to be a basal constitutive NR activity that could be used by the cell during the transition to anaerobiosis in the presence of nitrate to generate enough energy to allow for the synthesis of the major anaerobiosis- and nitrate-responsive NR-A activity. This was a reasonable hypothesis given the available data (Barrett & Riggs, 1982; reviewed by Gennis & Stewart, 1996). However, in light of its now realized complex regulation and the fact that it is required for long-term-starvation survival, H₂O₂-inducible H₂O₂ resistance (Spector & Cubitt, 1992; Seymour *et al.*, 1996), C-starvation-inducible thermotolerance (Fig. 2) and acid tolerance (Fig. 3), this relatively simplistic role seems incomplete at best.

The exact physiological role of NR-Z activity is unclear at this time. However, the facts that the *stiA* and *sti-99* designated fusions were identified from two different screens for stress-responsive genetic loci, exhibit complex global and stress regulation and are expressed within MDCK epithelial cells suggest a key role in starved- and stressed-cell physiology. Furthermore, the probability that *narZYWV* resulted from a duplication of *narGHJI* in a common ancestor of *E. coli* and *Salmonella* and the fact that it has been functionally conserved while its regulation has diverged from that of NR-A support the hypothesis of a key physiological role for the NR-Z activity in the cell at some time during its life cycle (Barrett & Riggs, 1982; Bonnefoy & DeMoss, 1994; Gennis & Stewart, 1996).

The reason why NR-Z is necessary for resistance to H₂O₂ only in growing cells exposed to exogenously added H₂O₂ but is needed for acid and thermal tolerance only in C-starved cells probably lies in its as yet unidentified physiological function and the redundancy that is common among stress response functions. The fact that σ^S is needed not only for stress-specific adaptive stress responses but also for starvation-induced stress responses supports the idea of redundancy.

Other respiratory enzymes or components have also been associated with stationary-phase and stressed-cell physiology. In *E. coli*, mutants in NADH dehydrogenase

I are unable to develop the GASP (growth advantage in stationary-phase) phenotype. The GASP phenotype is thought to result from mutations that occur in stationary-phase cells (i.e. prolonged growth in LB broth medium) that provide a growth advantage to the cell, allowing them to take over the stationary-phase culture (Zambrano *et al.*, 1993; Zambrano & Kolter, 1993). In *Salmonella*, mutants in the energy-conserving NADH dehydrogenase, i.e. *nuo* mutants, exhibit defective energy-dependent proteolysis during C starvation (Archer *et al.*, 1993). In *E. coli*, both the *hyaABCDE* (hydrogenase I) and the *cbdAB* (cytochrome *bd*-II oxidase; also known as *cyxAB*) operons are anaerobiosis inducible, C- and P-starvation inducible, and stationary-phase inducible in rich medium. Furthermore, the induction of both *hya* and *cbdAB* upon entry into stationary-phase is under σ^S control. Also, the C-starvation induction of *hya* is σ^S dependent while the C-starvation induction of *cbdAB* is indirectly σ^S regulated through the *appY* gene (Atlung *et al.*, 1997). Interestingly, *E. coli* cells deficient in another cytochrome *bd* oxidase, encoded by *cydAB*, are hypersensitive to H_2O_2 and high temperatures (Wall *et al.*, 1992; Goldman *et al.*, 1996), similar to *narZYWV* mutants. Recently, the *cydAB*-encoded cytochrome *bd* oxidase has been implicated in intracellular survival and virulence of *Shigella flexneri* (Way *et al.*, 1999). Thus, there is some precedent for a link between respiratory enzymes, stress responses and survival, as well as virulence potential.

It would seem unlikely that NR-Z would have a role in respiration and energy production in the starved or stressed cell. The reason for this is that its regulation and roles in stress resistance have been studied in well aerated cultures where oxygen should be the much preferred electron acceptor and nitrate should be scarce at best, since none has been added to the culture media. However, the possibility that it may use an alternative electron acceptor(s), e.g. nitric oxide or NO, has not been ruled out and is under study in our laboratory.

NR-Z may be important in the defence of *Salmonella* against so-called bacterial ageing (Nyström *et al.*, 1996; Dukan & Nyström, 1998). Nyström and colleagues present a convincing case that oxidation of proteins is a key correlate to cell ageing and possibly cell death in stationary-phase cells. They also demonstrate that stationary-phase induced protein oxidation is enhanced in *oxyR* and *rpoS* mutant cells. In this context, it is interesting that *narZYWV* expression is σ^S dependent and H_2O_2 inducible. As such, NR-Z may play a role in detoxifying reactive oxygen species, or other by-products, that might be generated under conditions such as long-term starvation, presence of H_2O_2 , high temperature or acidic pH. Its membrane location suggests that in such a role it may be involved in the repair of membrane damage or detoxification of products formed during aerobic metabolism (i.e. electron transport) at the inner membrane under various stress conditions. However, this has yet to be demonstrated. Studies designed to dissect the complex regulatory region of *narZYWV* and to determine its function(s) during

starvation and stress conditions are currently under way in our laboratory.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Julio Turrens for technical assistance and helpful discussions, Marc Woodland for automated sequencing help, and Paul Everest, Gill Douce and the staff of Central Biological Services for assistance with *in vivo* studies. We would also like to thank Dr Paul Gulig for supplying intracellular salts medium.

Portions of the work presented here were funded by grants from the National Institutes of Health (NIH) grant no. GM47628-01 and the University of South Alabama Research Council (to M.P.S.), NIH grant no. GM48017 (to J.W.F.) and the Medical Research Council of Canada (to B.B.F.). M.J.P. and G.D. would also like to thank the Wellcome Trust for supporting a Research Leave Fellowship for M.J.P.

REFERENCES

- Archer, C. D., Wang, X. & Elliott, T. (1993). Mutants defective in the energy-conserving NADH dehydrogenase of *Salmonella typhimurium* identified by a decrease in energy-dependent proteolysis after carbon starvation. *Proc Natl Acad Sci USA* **90**, 9877–9881.
- Atlung, T., Knudsen, K., Lotte, H. & Brøndsted, L. (1997). Effects of σ^S and the transcriptional activator AppY on induction of the *Escherichia coli* *hya* and *cbdAB-appA* operons in response to carbon and phosphate starvation. *J Bacteriol* **179**, 2141–2146.
- Babior, B. M. (1992). The respiratory burst oxidase. *Adv Enzymol Relat Areas Mol Biol* **65**, 49–95.
- Barrett, E. L. & Riggs, D. L. (1982). Evidence for a second nitrate reductase activity that is distinct from the respiratory enzyme in *Salmonella typhimurium*. *J Bacteriol* **150**, 563–571.
- Blasco, F., Iobbi, C., Ratouchniak, J., Bonnefoy, V. & Chippaux, M. (1990). Nitrate reductases of *Escherichia coli*: sequence of the second nitrate reductase and comparison with that encoded by the *narGHJI* operon. *Mol Gen Genet* **222**, 104–111.
- Bonnefoy, V. & DeMoss, J. A. (1994). Nitrate reductases in *Escherichia coli*. *Antonie Leeuwenhoek* **66**, 47–56.
- Brown, M. R. W. & Williams, P. (1985). The influence of environment on envelope properties affecting survival of bacteria in infections. *Annu Rev Microbiol* **39**, 527–556.
- Cashel, M., Gentry, D. R., Hernandez, V. J. & Vinella, D. (1996). The stringent response. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 1458–1496. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Castilho, B. A., Olfson, P. & Casadaban, M. J. (1984). Plasmid insertion mutagenesis and *lac* gene fusions with mini-Mu bacteriophage transposons. *J Bacteriol* **158**, 488–495.
- Chan, R. K., Botstein, D., Watanabe, T. & Ogata, Y. (1972). Specialized transduction of tetracycline resistance by phage P22 in *Salmonella typhimurium*. II. Properties of a high transducing lysate. *Virology* **50**, 883–898.
- Davis, R. W., Botstein, D. & Roth, J. R. (1980). *Advanced Bacterial Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Dukan, S. & Nyström, T. (1998). Bacterial senescence: stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon. *Genes Dev* **12**, 3431–3441.

- Fang, F. C., Libby, S. J., Buchmeier, N. A., Loewen, P. C., Switala, J., Harwood, J. & Guiney, D. G. (1992). The alternative σ factor KatF (RpoS) regulates *Salmonella* virulence. *Proc Natl Acad Sci USA* **89**, 11978–11982.
- Finlay, B. B. & Falkow, S. (1989). *Salmonella* as an intracellular parasite. *Mol Microbiol* **3**, 1833–1841.
- Foster, J. W. & Spector, M. P. (1986). Phosphate-starvation regulon of *Salmonella typhimurium*. *J Bacteriol* **166**, 666–669.
- Foster, J. W. & Spector, M. P. (1995). How *Salmonella* survive against the odds. *Annu Rev Microbiol* **49**, 145–174.
- Garcia del Portillo, F., Foster, J. W., Maguire, M. E. & Finlay, B. B. (1992). Characterization of the micro-environment of *Salmonella typhimurium*-containing vacuoles within MDCK epithelial cells. *Mol Microbiol* **6**, 3289–3297.
- Gennis, R. B. & Stewart, V. (1996). Respiration. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 217–261. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Goldman, B. S., Gabbert, K. K. & Kranz, R. G. (1996). The temperature-sensitive growth and survival phenotypes of *Escherichia coli* *cydDC* and *cydAB* strains are due to deficiencies in cytochrome *bd* and are corrected by exogenous catalase and reducing agents. *J Bacteriol* **178**, 6348–6351.
- Harder, W. & Dijkhuizen, L. (1983). Physiological responses to nutrient limitation. *Annu Rev Microbiol* **37**, 1–23.
- Hengge-Aronis, R. (1993). The role of *rpoS* in early stationary-phase gene regulation in *Escherichia coli* K12. In *Starvation in Bacteria*, pp. 171–200. Edited by S. Kjelleberg. New York: Plenum.
- Hengge-Aronis, R. (1996). Regulation of gene expression during entry into stationary phase. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 1497–1512. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Hoiseth, S. K. & Stocker, B. A. D. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**, 238–239.
- Iobbi-Nivol, C., Santini, C. L., Blasco, F. & Giordano, G. (1990). Purification and further characterization of the second nitrate reductase of *Escherichia coli* K-12. *Eur J Biochem* **188**, 679–687.
- Jenkins, D. E., Schultz, J. E. & Matin, A. (1988). Starvation-induced cross-protection against heat or H_2O_2 challenge in *Escherichia coli*. *J Bacteriol* **170**, 3910–3914.
- Koch, A. L. (1971). The adaptive response of *Escherichia coli* to a feast and famine existence. *Adv Microb Physiol* **6**, 147–217.
- Lange, R. & Hengge-Aronis, R. (1991). Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol Microbiol* **5**, 49–59.
- Lee, I. S., Slonczewski, J. L. & Foster, J. W. (1994). A low-pH inducible stationary-phase acid tolerance response in *Salmonella typhimurium*. *J Bacteriol* **176**, 1422–1426.
- Loewen, P. C. & Hengge-Aronis, R. (1994). The role of the sigma factor σ^S (KatF) in bacterial global regulation. *Annu Rev Microbiol* **48**, 53–80.
- McCann, M. P., Fraley, C. D. & Matin, A. (1991). The putative σ factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*. *J Bacteriol* **173**, 4188–4194.
- McLeod, G. I. & Spector, M. P. (1996). Starvation- and stationary-phase-induced resistance to the antimicrobial peptide polymyxin B in *Salmonella typhimurium* is RpoS (σ^S) independent and occurs through both *phoP*-dependent and -independent pathways. *J Bacteriol* **178**, 3683–3688.
- Mahan, M. J., Tobias, J. W., Slauch, J. M., Hanna, P. C., Collier, J. R. & Mekalanos, J. J. (1995). Antibiotic based selection for bacterial genes that are specifically induced during infection of a host. *Proc Natl Acad Sci USA* **92**, 669–673.
- Maloy, S. R. (1990). *Experimental Techniques in Bacterial Genetics*. Boston, MA: Jones & Bartlett.
- Matin, A. (1991). The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. *Mol Microbiol* **5**, 3–10.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Miller, J. H. (1992). *A Short Course in Bacterial Genetics: a Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Moriarty, D. J. W. & Bell, R. T. (1993). Bacterial growth and starvation in aquatic environments. In *Starvation in Bacteria*, pp. 25–53. Edited by S. Kjelleberg. New York: Plenum.
- Morita, R. Y. (1988). Bioavailability of energy and its relationship to growth and starvation survival in nature. *Can J Microbiol* **34**, 436–441.
- Mulvey, M. R. & Loewen, P. C. (1989). Nucleotide sequence of *katF* of *Escherichia coli* suggests KatF protein is a novel σ transcription factor. *Nucleic Acids Res* **17**, 9979–9991.
- Neidhardt, F. C., Bloch, P. L. & Smith, D. F. (1974). Culture medium for enterobacteria. *J Bacteriol* **119**, 736–747.
- Nyström, T., Larsson, C. & Gustafsson, L. (1996). Bacterial defense against aging: role of the *Escherichia coli* ArcA regulator in gene expression, readjusted energy flux and survival during stasis. *EMBO J* **15**, 3219–3228.
- O'Neal, C. R., Gabriel, W. M., Turk, A. K., Libby, S. J., Fang, F. C. & Spector, M. P. (1994). RpoS is necessary for both the positive and negative regulation of starvation survival genes during phosphate, carbon, and nitrogen starvation in *Salmonella typhimurium*. *J Bacteriol* **176**, 4610–4616.
- Parks, C. L., Chang, L. S. & Shenk, T. (1991). A polymerase chain reaction mediated by a single primer: cloning of genomic sequences adjacent to a serotonin receptor protein coding region. *Nucleic Acids Res* **19**, 7155–7160.
- Rosenthal, A., Coutelle, O. & Craxton, M. (1993). Large-scale production of DNA sequencing template by microtitre format PCR. *Nucleic Acids Res* **21**, 173–174.
- Roszak, D. B. & Colwell, R. R. (1987). Survival strategies of bacteria in the natural environment. *Microbiol Rev* **51**, 365–379.
- Seymour, R. L., Mishra, P. V., Khan, M. A. & Spector, M. P. (1996). Essential roles of core starvation-stress response loci in carbon-starvation-inducible cross-resistance and hydrogen peroxide-inducible adaptive resistance to oxidative challenge in *Salmonella typhimurium*. *Mol Microbiol* **20**, 497–505.
- Spector, M. P. (1990). Gene expression in response to multiple nutrient-starvation conditions in *Salmonella typhimurium*. *FEMS Microbiol Ecol* **74**, 175–184.
- Spector, M. P. (1998). The starvation-stress response (SSR) of *Salmonella*. *Adv Microb Physiol* **40**, 233–279.
- Spector, M. P. & Cubitt, C. L. (1992). Starvation-inducible loci of *Salmonella typhimurium*: regulation and roles in starvation survival. *Mol Microbiol* **6**, 1467–1476.
- Spector, M. P. & Foster, J. W. (1993). Starvation-stress response (SSR) of *Salmonella typhimurium*: gene expression and survival

during nutrient starvation. In *Starvation in Bacteria*, pp. 201–224. Edited by S. Kjelleberg. New York: Plenum.

Spector, M. P., Aliabadi, Z., Gonzalez, T. & Foster, J. W. (1986). Global control in *Salmonella typhimurium*: two-dimensional gel electrophoretic analysis of starvation-, anaerobiosis-, and heat-shock-inducible proteins. *J Bacteriol* **168**, 420–424.

Spector, M. P., Park, Y. K., Tirgari, S., Gonzalez, T. & Foster, J. W. (1988). Identification and characterization of starvation-regulated genetic loci in *Salmonella typhimurium* by using Mud-directed *lacZ* operon fusions. *J Bacteriol* **170**, 345–351.

Spector, M. P., DiRusso, C. C., Pallen, M. J., Garcia del Portillo, F., Dougan, G. & Finlay, B. B. (1999). The medium-/long-chain fatty acyl-CoA dehydrogenase (*fadF*) gene of *Salmonella typhimurium* is a phase 1 starvation-stress response (SSR) locus. *Microbiology* **145**, 15–31.

Storz, G. & Altuvia, S. (1994). OxyR regulon. *Methods Enzymol* **234**, 217–223.

Storz, G., Tartaglia, L. A. & Ames, B. N. (1990). Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science* **248**, 189–194.

Tanaka, K., Takayanagi, Y., Fujita, N., Ishihama, A. & Takahashi, H. (1993). Heterogeneity of the principal σ factor in *Escherichia coli*: the *rpoS* gene product, σ^{38} , is a second principal σ factor of RNA polymerase in stationary-phase *Escherichia coli*. *Proc Natl Acad Sci USA* **90**, 3511–3515.

Valdivia, R. H. & Falkow, S. (1997). Probing bacterial gene expression within host cells. *Trends Microbiol* **5**, 360–363.

Wall, D., Delaney, J. M., Fayet, O., Lipinska, B., Yamamoto, T. & Georgopoulos, C. (1992). *arcA*-dependent thermal regulation and extragenic suppression of the *Escherichia coli* cytochrome *d* operon. *J Bacteriol* **174**, 6554–6562.

Way, S. S., Sallustio, S., Magliozzo, R. S. & Goldberg, M. B. (1999). Impact of either increased or decreased levels of cytochrome *bd* expression on *Shigella flexneri* virulence. *J Bacteriol* **181**, 1229–1237.

Wilson, J. A., Doyle, T. J. & Gulig, P. A. (1997). Exponential-phase expression of *spvA* of the *Salmonella typhimurium* virulence plasmid: induction in intracellular salts medium and intracellularly in mice and cultured mammalian cells. *Microbiology* **143**, 3827–3839.

Zambrano, M. M. & Kolter, R. (1993). *Escherichia coli* mutants lacking NADH dehydrogenase I have a competitive disadvantage in stationary phase. *J Bacteriol* **175**, 5642–5647.

Zambrano, M. M., Siegele, D. A., Almirón, M., Tormo, A. & Kolter, R. (1993). Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. *Science* **259**, 1757–1760.

Received 27 April 1999; revised 28 June 1999; accepted 23 July 1999.