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

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**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 σs-dependent SSR locus that is phosphate-starvation, nitrogen-starvation and H2O2 inducible, positively regulated by (p)pGpp 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 (*narZ*Y*WV*) 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 *narZ* 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 *narZ*Y*WV* 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

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**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 H$_2$O$_2$ 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 (Castillo 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, $\sigma^S$ or $\sigma^{26}$ (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 H$_2$O$_2$-inducible H$_2$O$_2$ resistance (Seymour et al., 1996; M. P. Spector, unpublished). The H$_2$O$_2$-inducible H$_2$O$_2$ 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 H$_2$O$_2$ in exponential-phase cells. In the presence of H$_2$O$_2$, *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 H$_2$O$_2$-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 $\sigma^S$ 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 *narZ*Y*WV* 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 aerobiciosis (Barrett & Riggs, 1982; Bonnefoy & DeMoss, 1994; reviewed by Gennis & Stewart, 1996). In fact, *narZ*Y*WV* was repressed during anaerobic growth conditions by the *fnr* gene product, Fnr. We also report that NR-Z was required for C-starvation-inducible thermostolerance 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 LD$_{50}$ 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., 1987; reviewed by Gennis & Stewart, 1996). MS medium nonlimiting in glucose, phosphate and
flanking the unit (Millipore). The filtrate was collected, ethanol precipi-
tation, and DNA sequencing of cells while MS medium limiting in glucose (0
*Kan* was used to generate C-starved cells. The rich medium
loC was used to generate C-starved cells. The rich medium

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype (pertinent phenotype)*</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL1344</td>
<td><em>bisG46</em> (virulent <em>Salmonella typhimurium</em>)</td>
<td>Hoiseth &amp; Stocker (1981)</td>
</tr>
<tr>
<td>ST63</td>
<td>SL1344 <em>zar2</em> [st99]::<em>Mu</em>J (<em>lac Kan</em>)</td>
<td>This study</td>
</tr>
<tr>
<td>ST66</td>
<td>SL1344 <em>narZ1</em> [stai1]::<em>Mu</em>J (<em>lac Kan</em>)</td>
<td>Specter et al. (1988); O’Neal et al. (1994)</td>
</tr>
<tr>
<td>SMS438</td>
<td>SL1344 *roo56::<em>Omega</em>8</td>
<td>Fang et al. (1992); O’Neal et al. (1994)</td>
</tr>
<tr>
<td>SMS575</td>
<td>ST66 <em>hir3</em>::<em>Tn10</em> (<em>Tet</em>)</td>
<td>This study; J. Foster†</td>
</tr>
<tr>
<td>SMS643</td>
<td>SL1344 <em>narGHJ1715</em>::<em>Mu</em>J (<em>lac Kan</em>)</td>
<td>This study; V. Stewart‡</td>
</tr>
</tbody>
</table>

* Kan*, kanamycin resistance; *Amp*, ampicillin resistance; *Tet*, 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* *Mu*J insertion sites. DNA adjacent to the *stiA*::*Mu*J insertion site was cloned by digesting chromosomal DNA isolated from strain ST66 with *Sal*I, using protocols supplied by the manufacturer (Promega). *Sal*I was chosen because there is a single site within *Mu*J, about 2.5 kb from the left end of *Mu*J and just upstream from the Kan gene, so that *Sal*I restriction fragments of 2.5 kb or greater would contain the Kan gene and remaining *Mu*J DNA plus any chromosomal DNA adjacent to the insertion site. *Sal*I 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 *Sal*I-digested pACYC184, ligated with T4 ligase and electrotransformed into electrocompetent recipient cells, selecting for Kan gene (target DNA) and Cam<sup>®</sup> (vector DNA). Kan<sup>®</sup> Cam<sup>®</sup> transforms were picked, and their plasmids were isolated using Wizard Mini-prep columns (Promega), digested with *Sal*I 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 *Mu*J (Mu<sup>L</sup>; GenBank accession no. M10190) into adjacent chromosomal DNA. However, exponential amplification of the *Mu*J/chromosome junction site only occurs if the Mu<sup>L</sup> 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 *Mu*J 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 hypercompetent 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 *A<sub>600</sub>*. Cells were grown in MS hiPCN to *A<sub>600</sub>* 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 A₄₅₀ 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 ÷ (A₄₅₀ × 15 min × 0.5 or 1.5 ml cells); where A₄₅₀ is A₄₅₀ of culture at the point of assay. An NR unit equals 1 nmol NO₂⁻ formed min⁻¹ (A₄₅₀ × 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 37 °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 × 10⁸ 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; García 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 (~10⁴ 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 % (w/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 (García 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 OD₆₀₀ 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 σ⁷₀-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
**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(YWW)**

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pertinent genotype</th>
<th>NR activity (NR units)</th>
<th>Aerobiosis, no nitrate, exponential phase</th>
<th>Anaerobiosis, 50 mM nitrate, exponential phase</th>
<th>Aerobiosis, no nitrate, 3h C starved</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL1344</td>
<td>narZYWV⁺ narGHJI⁺</td>
<td>0.24 ± 0.01</td>
<td>3.44 ± 0.33</td>
<td>1.21 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>SMS643</td>
<td>narZYWV⁺ narGHJI[1715::MudJ]</td>
<td>0.23 ± 0.02</td>
<td>0.22 ± 0.01</td>
<td>1.15 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>ST66</td>
<td>narZ1::MudJ narGHJI⁺</td>
<td>0.24 ± 0.03</td>
<td>2.82 ± 0.55</td>
<td>0.21 ± 0.11</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Role of Fnr in narZYWV operon expression in S. typhimurium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pertinent genotype</th>
<th>β-Galactosidase activity (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aerobiosis, exponential phase</td>
</tr>
<tr>
<td>ST66</td>
<td>narZ1::lac</td>
<td>9.88 ± 1.65</td>
</tr>
<tr>
<td>SMS75</td>
<td>narZ1::lac fnr::Tn10</td>
<td>25.25 ± 3.85</td>
</tr>
</tbody>
</table>

Values are means ± SEM of at least three separate experiments.

The results presented in Table 3 further support that the indicated). Analyses performed in this study (unless otherwise indicated). 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 aerobically 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 aerobically growing cells (compare anaerobiosis, exponential-phase ST66 and SMS75 in Table 3) to about the level observed in aerobically growing wild-type cells (compare anaerobiosis, exponential-phase ST66 and SMS75 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 SMS75 in Table 3). Thus, the S. typhimurium narZYWV 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, 3h C-starved ST66 and SMS75 in Table 3).

Role of the narZYWV 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...
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 detected after only 10 min at pH 2.8. Interestingly, the narZ knock-out mutation had no 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 narZ(YWV) 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 narZ(YWV) was induced 19.58 ± 5.72-fold (mean ± SEM, n = 4) within MDCK epithelial cells. Furthermore, the narZ1::lac fusion was found to be induced 15.8 ± 0.82-fold (mean ± 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

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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 LD₉₀ 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 LD₉₀ values. Mice were monitored for 28 d. The LD₉₀ values were calculated to be approximately 10⁴·₅ for SL1344 and 10⁴·₄ for ST66 (a little more than 10-fold higher). Thus, the narZ knock-out mutant exhibited a higher LD₉₀ 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 sti 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) σ⁵-dependent, (c) positively regulated by (pppGpp 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 thermo-tolerance (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 σ⁵ 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 am 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. *mut* mutants, exhibit defective energy-dependent proteolysis during C starvation (Archer et al., 1993). In E. coli, both the *byaABCDEF* (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 *bya* and *cbdAB* upon entry into stationary-phase is under σ² control. Also, the C-starvation induction of *bya* is σ² dependent while the C-starvation induction of *cbdAB* is indirectly σ² 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 H₂O₂ 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. Moreover, the induction of both *bya* and *cbdAB* upon entry into stationary-phase is under σ² control. Also, the C-starvation induction of *bya* is σ² dependent while the C-starvation induction of *cbdAB* is indirectly σ² 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 H₂O₂ 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.

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 *rho* mutant cells. In this context, it is interesting that *narZYWV* expression is σ² dependent and H₂O₂ 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 H₂O₂, 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.

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