σS-Dependent carbon-starvation induction of *pbpG* (PBP 7) is required for the starvation-stress response in *Salmonella enterica* serovar Typhimurium


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Carbon-energy source starvation is a commonly encountered stress that can influence the epidemiology and virulence of *Salmonella enterica* serovars. *Salmonella* responds to C-starvation by eliciting the starvation-stress response (SSR), which allows for long-term C-starvation survival and cross-resistance to other stresses. The *stiC* locus was identified as a C-starvation-inducible, σS-dependent locus required for a maximal SSR. We report here that the *stiC* locus is an operon composed of the *yohC* (putative transport protein) and *pbpG* (penicillin-binding protein-7/8) genes. *yohC pbpG* transcription is initiated from a σS-dependent C-starvation-inducible promoter upstream of *yohC*. Another (σS-independent) promoter, upstream of *pbpG*, drives lower constitutive *pbpG* transcription, primarily during exponential phase. C-starvation-inducible *pbpG* expression was required for development of the SSR in 5 h, but not 24 h, C-starved cells; *yohC* was dispensable for the SSR. Furthermore, the *yohC pbpG* operon is induced within MDCK epithelial cells, but was not essential for oral virulence in BALB/c mice. Thus, PBP 7 is required for physiological changes, occurring within the first few hours of C-starvation, essential for the development of the SSR. Lack of PBP 7, however, can be compensated for by further physiological changes developed in 24 h C-starved cells. This supports the dynamic overlapping and distinct nature of resistance pathways within the *Salmonella* SSR.

INTRODUCTION

A common stress encountered by *Salmonella* within numerous host and non-host microenvironments is starvation for a carbon-energy (C)-source (Koch, 1971; Brown & Williams, 1985; Fang et al., 1992; Foster & Spector, 1995; Spector, 1998; Spector et al., 1999b). The morphologic and physiological changes resulting from C-starvation are called the starvation-stress response (SSR). The SSR functions to provide long-term C-starvation survival (LT-CSS) and C-starvation-inducible...
(CSI) cross-resistance mechanisms to the bacteria. In *Salmonella* Typhimurium, core SSR genes are required for maximal/wild-type development of the SSR. In both *Escherichia coli* and *S. Typhimurium*, subsets of SSR genes are regulated by one or more of three sigma factors encoded by *rpoS* (*σ^S^* or σ^38^*), *rpoE* (*σ^E^* or σ^34^*) and *rpoD* (*σ^D^* or σ^26^*) (Jenkins et al., 1988; McCann et al., 1991; Fang et al., 1992; Spector & Cubitt, 1992; Tanaka et al., 1993; Loewen & Henge-Aronis, 1994; O’Neal et al., 1994; Seymour et al., 1996; McLeod & Spector, 1996; Spector, 1998; Spector et al., 1999a, b; Kenyon et al., 2002; Bang et al., 2005). In *S. Typhimurium*, neither *rpoS* nor *rpoE* are essential genes, but null mutants in either or both show significantly reduced LT-CSS, CSI cross-resistance and mouse virulence (Fang et al., 1992; O’Neal et al., 1994; Humphreys et al., 1999; Kenyon et al., 2002; Testerman et al., 2002; Kazmierczak et al., 2005; Rowley et al., 2006).

In previous studies (Spector, 1990; Spector et al., 1988; Spector et al., 1986; Spector & Cubitt, 1992; Spector & Foster, 1993; O’Neal et al., 1994; Seymour et al., 1996), a *S. Typhimurium* *stiC2*::*MudJ* (*lac* Km^R^) insertion was shown to be C-, phosphate (P)-, and nitrogen (N)-starvation-inducible in a *σ^S^*-dependent manner. In addition, *stiC2*::*lac* expression increased when intracellular levels of NAD fell to growth-limiting levels in the cell. The *stiC2*::*lac* fusion is negatively controlled by the cAMP- CRP complex and positively controlled by ppGpp during nutrient replete and depleted conditions, respectively.

Furthermore, the *stiC2*::*MudJ* insertion mutant is defective in LT-CSS and CSI cross-resistance to hydrogen peroxide. The *stiC* locus, therefore, meets the criteria of a core SSR gene.

Entry into stationary-phase for many rod-shaped bacteria results in a concomitant morphological change to smaller, more spherical cells, a transition influenced by the *rpoS* status of the cell. This suggested that *σ^S^* plays a role in controlling cell-wall synthesis in non-growing cells. Likely targets of *σ^S^* control are penicillin-binding proteins or PBPs, the targets of β-lactam antimicrobics. *σ^S^* was found to downregulate PBP 3 expression and upregulate PBP 6 expression via increased *bolA* gene expression in *E. coli* grown to stationary-phase in LB medium (LB-stationary-phase) (Dougherty & Pucci, 1994). Dougherty & Pucci (1994) also showed that other high-molecular-mass PBPs decrease in LB-stationary-phase, but through *rpoS*-independent mechanisms. It is known that non-growing cells are resistant to killing by most β-lactam antimicrobics. In contrast, Tuomanen & Schwartz (1987) reported that the low-molecular-mass PBP 7 [and its proteolytic derivative PBP 8, an artefact of cleavage of PBP 7 by the OmpT protease (Henderson et al., 1994)] binds to β-lactam antimicrobics capable of lysing non-growing (lysine-starved) *E. coli* cells. This suggests an important role for PBP 7 in the non-growing (e.g. starving) cell. However, this has not been reported or characterized further. PBP 7 possesses a DD-endopeptidase activity, which hydrolyses the D-diaminopimelate-D-alanine bonds in high-molecular-mass peptidoglycan, but not in isolated muropeptide dimers (Romeis & Höltje, 1994). Based on PBP 7’s function to break the peptide cross-bridge between two glycan chains, it is proposed to play a role in cell-wall remodelling (Romeis & Höltje, 1994).

Recently, PBP 4 endopeptidase activity has been implicated, along with three periplasmic amidases, AmA, AmB, and AmC, in daughter-cell separation during exponential-phase growth in LB medium. In this study, it was also shown that PBP 7 played a minor or secondary role in this process, since the loss of PBP 7 function had a detectable effect only in the absence of PBP 4 activity (Priyadarshini et al., 2006).

We report here that the *stiC2*::*MudJ* (*lac*) insertion lies within the *S. Typhimurium* *yohC* homologue, and that *yohC* and the downstream *pbpG* gene form a *σ^S^*-dependent CSI operon. We also showed that a separate *pbpG*-specific transcript is expressed constitutively in exponential-phase (growing) cells. Furthermore, we demonstrated that CSI levels of only PBP 7, but not YohC, are conditionally required for maximal/wild-type development of the SSR.

**METHODS**

**Bacterial strains, plasmids, primers, and phage/transductions used.** Bacterial strains, plasmids and oligonucleotide primers are listed in Table 1. Primer sequences were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were synthesized commercially (Invitrogen). Transductions were performed with the high-transducing derivative of P22 bacteriophage, P22 HT 105/1 int (HT phage) (Chan et al., 1972), and determined to be non-lysogens (Davis et al., 1980; Maloy, 1989).

**Culture media, supplements and antibiotics used.** The rich media used were LB broth and agar (Difco). The minimal media used were modified MOPS-buffered salts (MS)-based media (Neidhart et al., 1974), as described previously (Spector & Cubitt, 1992). MS medium with 0.4 % (w/v) glucose (MS hiC) or 0.03 % (w/v) glucose (MS loC) was used to generate exponential-phase cells and C-starved cells, respectively. Histidine was used at 0.2 mM, as needed. Kanamycin (Km), chloramphenicol (Cm), ampicillin (Ap) and tetracycline (Tc) were added, as needed, at final concentrations of 50 μg ml^−1^, 50 μg ml^−1^, 30 μg ml^−1^, and 20 μg ml^−1^ (LB) or 10 μg ml^−1^ (MS media), respectively.

**Growth and starvation conditions.** Desired strains were grown overnight in MS hiC medium at 37 °C with shaking and diluted 1:100 into fresh MS hiC or fresh MS loC medium, and incubated with aeration at 37 °C to generate exponential-phase, 5 h C-starved and 24 h C-starved cells, respectively (Seymour et al., 1996; Spector et al., 1999a, b). Growth was monitored by measuring optical density at 600 nm (OD_600). These cells were then used to (i) isolate whole cell RNA for RT–PCR, Northern blot, and/or transcription start point (TSP) mapping analyses, and (ii) to assay for β-galactosidase activity or (iii) perform desired challenge assays.

**Challenge assays.** Exponential-phase, 5 h and 24 h C-starved cells were diluted 1:100 and challenged in MS buffer (i) containing 15 mM H_2O_2 for 40 min, (ii) pre-heated at 55 °C for 16 min and (iii) at pH 3.0 for 60 min. At pre-determined time points an aliquot was removed, serially diluted in tenfold increments and plated onto LB agar plus antibiotic, as needed. Survival was calculated as percentage
Table 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strains, plasmids and primers</th>
<th>Relevant genotype or phenotype information*</th>
<th>Reference or source</th>
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<tr>
<td><strong>Bacterial strains</strong></td>
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<tr>
<td>ST68</td>
<td>SL1344 stcC1::MudI (lac Km(^{-}))</td>
<td>Spector &amp; Cubitt, (1992)</td>
</tr>
<tr>
<td>LB5000</td>
<td>LT-2 lab52 metaA22 metE551 trpD2 leu hisL LT hisD5 SA hisD8B</td>
<td>Bullas &amp; Ryu, (1983)</td>
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<td>SMS758</td>
<td>SL1344/pRS1274 (Ap(^{+}))</td>
<td>This work</td>
</tr>
<tr>
<td>SMS818</td>
<td>SL1344/pKS25 (Ap(^{+}))</td>
<td>This work</td>
</tr>
<tr>
<td>SMS819</td>
<td>SL1344/pKS26 (Ap(^{+}))</td>
<td>This work</td>
</tr>
<tr>
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<td>This work</td>
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<td>SMS923</td>
<td>SL1344 ΔyohC18 (Km(^{-}); SMS863 with T-Km(^{-}) cassette removed)</td>
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<td>EC33</td>
<td><em>Escherichia coli</em> K-12 (Δ(^{\text{K}})) (Stanford strain)</td>
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<td><strong>Plasmids</strong></td>
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<td>pKD4</td>
<td>Vector carrying the FRT-T-Km(^{-}}-FRT cassette (Ap(^{+}))</td>
<td>Datsenko &amp; Wanner, (2000)</td>
</tr>
<tr>
<td>pKD46</td>
<td>Ts (30 °C) replicon; encodes arabinose-inducible β, γ and Exo proteins of β-Red recombination system (Ap(^{+}))</td>
<td>Datsenko &amp; Wanner, (2000)</td>
</tr>
<tr>
<td>pCP20</td>
<td>Ts (30 °C) replicon carrying Flp recombinase gene (Ap(^{+}) Cm(^{+}))</td>
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<td>Simons et al., (1987)</td>
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<td><strong>Primer Oligonucleotide sequence</strong></td>
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<td>PR7</td>
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</tr>
<tr>
<td>pbpGREV</td>
<td>5'-CGTGTGGCCACAGGCTGGC-3'</td>
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</table>

*Km\(^{-}\), Kanamycin resistance; Ap\(^{+}\), ampicillin resistance; Tc\(^{+}\), tetracycline resistance; Sm\(^{-}\), streptomycin resistance; Cm\(^{-}\), chloramphenicol resistance.

by dividing the c.f.u. ml\(^{-}\) at each time point by the c.f.u. ml\(^{-}\) at the time zero point and then multiplying that number by 100. Data presented are means ± SEM for at least three separate experiments.

**Construction of promoter-lac fusion plasmids.** The yohD–yohC intergenic region (PR92 and PR95) and the yohC–pbpG intergenic region (PR93 and PR94) were PCR-amplified using Platinum High Fidelity PCR SuperMix (Invitrogen) and eventually cloned into the Smal site in front of a promoterless *lacZ* gene in the low copy number pRS1274 vector (Simons et al., 1987). The yohD–yohC intergenic region was cloned in both orientations to monitor promoter activity for both yohD (yohDp; pKS17) and yohC (yohCp; pKS25). The yohC–pbpG intergenic region was similarly amplified and cloned in the pbpG promoter orientation (pbpGp; pKS26).
\(\beta\)-Galactosidase assay. Desired strains were grown and starved as described above. At the appropriate times, cells were assayed for \(\beta\)-galactosidase activity. \(\beta\)-Galactosidase activity was expressed in Miller units (Miller, 1992). Data presented are means ± SEM for at least three separate trials.

Selenate/seelenite reduction assay. Desired strains were tested for the ability to reduce selenate and selenite to elemental selenium by scoring colonies perpendicular to (within 5 mm) a selenite- or selenate-saturated paper strip on a LB agar plate and a MS hiC agar plate. The sterile paper strips were saturated with 100 mM selenite or 100 mM selenate. Plates were incubated for 2 days at 37 °C and monitored for the production of a red deposit, indicating reduction of selenite/selenate to elemental selenium.

Determination of the stiC2::MudJ (lac Km') insertion site in the chromosome. The insertion site for the stiC2::MudJ (lac Km') was analysed as previously described (Parks et al., 1991; Rosenthal et al., 1993; Spector et al., 1999a, b).

Lambda-red mutagenesis. Construction of yohC null mutant (ΔyohC18::Δ-Km') was accomplished using a modified \(\lambda\)-red mutagenesis protocol (Datsenko & Wanner, 2000), as previously described (Humphreys et al., 1999; Kenyon et al., 2002), employing primers PR118 and PR119. PCR products were electroporated into freshly prepared electrocompetent ST276 cells using an E. coli Pulser (Bio-Rad). Km-resistant colonies were screened for the desired null mutation utilizing PCR. The ΔyohC17::Δ-Km' mutation was then transduced into SL1344 to generate SM8683.

Flp recombination protocol. The \(\Omega\)-Km' cassette was removed from SM8683 chromosome using Flp recombination as described by Datsenko & Wanner (2000). pCP20 (carries Flp recombination gene) was electroporated into freshly prepared electrocompetent SM863 cells using an E. coli Pulser (Bio-Rad). Km-resistant colonies were screened for the desired null mutation utilizing PCR. The ΔyohC18 mutation was then transduced into SL1344 to generate SM8683.

RNA isolation for Northern hybridization and RT-PCR. Total RNA was isolated from cell pellets of exponential-phase, 5 h C-starved and 24 h C-starved cells using RNAwiz (Ambion) and DNA contamination was removed by DNase I treatment (DNA-free kit; Ambion) according to the manufacturer’s protocols. Total RNA preparations were quantified, aliquoted and stored at -80 °C before being used for RT-PCR or Northern hybridization analyses.

Northern hybridization analysis. Total RNA from exponential-phase, 5 h C-starved and 24 h C-starved cells was analysed by Northern blotting using NorthernMax reagents and protocols (Ambion). Briefly, total RNA samples were separated by electrophoresis on a denaturing agarose gel and transferred to a Zeta-Probe GT nitrocellulose membrane (Bio-Rad) using a Bio-Rad model 785 vacuum blotter and related protocols (Bio-Rad). The RNA was cross-linked to the membrane using a GS Gene Linker UV Chamber (Bio-Rad). Blocking, probe hybridization, and washing steps were performed as described for the NorthernMax Kit (Ambion). Digoxigenin (DIG)-labelled probes were prepared by PCR incorporation of DIG-labelled nucleotides using the PCR DIG Probe Synthesis Kit (Roche Applied Science). Chemiluminescent detection of hybridized probes was accomplished with the DIG Wash and Block Buffer Set and the DIG Luminescent Detection Kit (Roche Applied Science). Images were obtained using the Amersham ECL mini-camera (Amersham Pharmacia Biotech). The approximate size of mRNA transcripts was determined by comparison to DIG-labelled RNA molecular mass markers run in a separate lane of the same gel (Roche Applied Science).

RT-PCR. RNA from exponential-phase, 5 h C-starved and 24 h C-starved cells was used as a template for RT-PCR using the SuperScript One-Step RT-PCR system with Platinum Taq according to the manufacturer’s protocols (Invitrogen). A Taq only (–RT) control reaction was set up as recommended by the manufacturer’s protocols (Invitrogen) using RNA from all three growth conditions. Primers specific for sequences within the 5’-end of yohC ORF (PR53) and the 5’-end of pbpG ORF (PR56) (Table 1) were used. RT-PCR products were analysed using agarose-TBE gel electrophoresis followed by ethidium bromide staining.

S1-nuclease mapping and primer extension analyses. Overnight cultures of desired strains were diluted 100-fold into 50 ml of fresh MS hiC or MS loC media and incubated at 37 °C with aeration to produce exponential-phase cells, 5 h C-starved cells or 24 h C-starved cells, respectively. Total RNA was prepared essentially as described by Kormanec (2001). S1-nuclease mapping and primer extension analysis was performed as described previously (Kormanec, 2001; Rezuchova et al., 2003; Skovierova et al., 2006). The protected DNA fragments and primer extension products were analysed on DNA sequencing gels together with G + A and T + C sequencing ladders derived from the end-labelled fragments (Maxam & Gilbert, 1980). The probes used for S1-nuclease mappings were as follows: (i) S1 probe 1 (for yohCp) was a 421 bp DNA fragment prepared by PCR using a 5' labelled yohCREV primer and unlabelled yohCFOR primer; (ii) S1 probe 2 (for yohDp) was a 421 bp DNA fragment prepared by PCR using a 5' labelled yohCFOR primer and unlabelled yohCREV primer; and (iii) S1 probe 3 (for pbpGp) was a 1126 bp DNA fragment prepared by PCR using a 5' labelled pbpGREV primer and unlabelled yohCFOR primer. In all PCRs, the S. Typhimurium SL1344 chromosomal DNA was used as a template. Oligonucleotides were labelled at the 5’ end with [\(^{32}\)P]ATP (1.665 x 10^4 Bq mmol\(^{-1}\), ICN Radiochemicals) and T4 polynucleotide kinase (New England Biolabs).

Sequence analysis. BLASTP and PSI-BLAST searches with the YohC and PbpG sequences were performed on the ViruloGenome server (http://www.vge.ac.uk). Multiple alignments were performed on the EBI’s CLUSTAL W server (http://www.ebi.ac.uk/clustalw/). The BoxShade server was used to shade the alignment (http://www.ch.embnet.org/software/BOX_form.html). Analyses of trans-membrane domains were performed using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). Relevant nucleotide sequences were retrieved using the NCBI’s Entrez server. Bacterial terminator analysis was done using FindTerm (http://www.softberry.com/)

Assay for expression in MDCK cells. Infection and assay for intracellular \(\beta\)-galactosidase expression was carried out as previously described (Finlay & Falkow, 1989; Garcia del Portillo et al., 1992; Spector et al., 1999b).

Virulence assays. Cultures of the S. enterica strains to be tested were grown and administered intra-gastrically to 6- to 8-week-old female BALB/c mice by oral gavage in a volume of 200 μl, as previously described (Spector et al., 1999b).

RESULTS AND DISCUSSION

The stiC::MudJ (lac Km') insertion lies in the yohC homologue of S. enterica

We previously reported that a cAMP-CRP-negatively regulated \(\sigma^S\)-dependent CSI gene locus designated stiC

http://mic.sgmjournals.org
was required for the SSR in S. Typhimurium (Spector et al., 1988; Specter & Cubitt, 1992; O’Neal et al., 1994; Seymour et al., 1996). We analysed the DNA adjacent to a stiC2::MudI insertion using single-primer PCR (SP-PCR) amplification and sequencing protocols (Spector et al., 1999a, b). BLAST searches, using the 332 nt sequenced, revealed that the insertion was after nt 117 (amino acid 39) within the STM2169 gene (GenBank accession no. AE008796, complement 18896–19483), an orthologue of the E. coli K-12 MG1655 yohC gene (accession no. U00096).

YohC of S. Typhimurium (YohCSTM) is a 195 aa protein (GenPept accession no. AAL21073) that has homologues in several other genome-sequenced γ-proteobacteria (BLASTP analysis; see sequence alignments in Supplementary Figure S1, available with the online version of this paper). YohCSTM is predicted to be an inner-membrane transport protein of the DUF1282 family (Pfam; http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF06930). TMHMM analysis strongly predicts an N-terminal cytoplasmic domain and five transmembrane domains (amino acids 33–55, 65–87, 108–130, 135–157 and 170–192), supporting a membrane location for YohCSTM. The N-terminal domain of YohCSTM, and its homologues, possesses multiple conserved histidine residues (six in YohCSTM), suggesting that this motif is involved in the function of YohCSTM and its homologues.

Bébien et al. (2002) reported that an E. coli yohC mutant is unable to reduce selenite to elemental selenium, but is able to reduce selenite to selenate, suggesting it is defective in selenate transport. We screened our strain for the ability to reduce selenate to elemental selenium, but is able to reduce selenite to elemental selenium, and its homologues, possesses multiple conserved histidine residues (six in YohCSTM), suggesting that this motif is involved in the function of YohCSTM and its homologues.

However, we cannot rule out a role in selenate transport.

**yohC and pbpG, located downstream of yohC, comprise a two-gene operon**

In Salmonella, yohC was 165 bp upstream of the STM2168 gene, a putative homologue of the E. coli pbpG gene encoding PBP7. The yohC and STM2168 genes are transcribed in the same direction on the chromosome (Fig. 1). STM2170, a homologue of the E. coli yohD gene encoding a putative member of the DedA family, was transcribed in the same direction on the chromosome.

**TMHMM and domain analyses of yohCSTM**

TMHMM and domain analyses of the STM2168 gene product reveals a 316 aa periplasmic protein possessing a putative N-terminal signal peptide sequence, the four conserved peptide motifs of PBPs (Henderson et al., 1994, 1995; Goffin & Ghuysen, 2002) and a lysine-lysine (KK) dipeptide representing the putative OmpT cleavage site (at residues 293–294) that produces PBP8 from PBP7 (Henderson et al., 1994) in E. coli. Thus, STM2168 seems to encode the S. Typhimurium PBP7.

Based on the ORF analysis of this region, we proposed that yohC and pbpG are co-expressed as an operon under certain conditions. To test this, RT-PCR analysis using RNA from exponential-phase, 5 h C-starved and 24 h C-starved cells, and primers complementary to yohC (PR53) and pbpG sequence (PR56) (Table 1; Fig. 2) was performed. A PCR product of approximately 1.5 kb was detected from RNA under all three conditions (Fig. 2). This indicated that a transcript covering both yohC and pbpG was synthesized under the three conditions tested. Although this method is not quantitative, the level of yohC–pbpG co-transcript-derived product was considerably higher in C-starved cells, implying the presence of higher levels of co-transcript in

![Fig. 1. Schematic illustrating the organization of the yohD yohC pbpG region of the Salmonella enterica chromosome. Predicted θ70-dependent promoters for yohD and pbpG and θ54-dependent promoter for the yohC pbpG operon are shown. ORFs are indicated by lower-case letters. Predicted ribosome-binding sites (RBS) are designated. The TSP for the individual genes is indicated by thick bent arrows.](Image 153 to 306)
C-starved cells compared to exponential-phase cells. This is supported by Northern hybridization and TSP analyses.

**The yohCp, but not pbpGp, is a σ^S-dependent cAMP-CRP-negatively regulated CSI promoter**

Results presented in Figs 3(a), 4(b) demonstrate that a yohC–pbpG co-transcript was induced in 5 h C-starved cells. The level of this yohC–pbpG co-transcript declined in 24 h C-starved cells, but was still detected at a higher level than in exponential-phase cells. The yohC–pbpG co-transcript was undetectable by Northern hybridization in exponential-phase cells (Fig. 3a), although it was detected using RT-PCR (Fig. 2). Data presented in Fig. 3(b) show that the yohC upstream promoter (yohCp) is CSI in a σ^S-dependent manner, and is negatively regulated by cAMP-CRP in exponential-phase cells. This agrees with previous results showing that the stiC2::MudJ (lac Km') fusion was CSI, σ^S-dependent and negatively regulated by cAMP-CRP (Spector et al., 1988; Spector & Cubitt, 1992; O’Neal et al., 1994).

A smaller yohC-specific transcript was also detected in both 5 h and 24 h C-starved cells Figs 3(a), 4(b). Both these transcripts disappeared in the stiC2::MudJ mutant (ST68; Fig. 3a). Comparison of Northern blot (Fig. 3a) and yohCp::lacZ (Fig. 3b) analyses in 24 h C-starved cells showed that the yohCp was still induced, whereas pbpG-containing transcripts were very low. This suggests that the yohC (specific) transcript was a product of transcription termination within the yohC–pbpG intergenic region and/or post-transcriptional processing removing the pbpG sequence. The latter scenario is supported by intermediate-sized bands detected between the yohC–pbpG co-transcript and yohC transcript that disappear in the stiC2::MudJ mutant. Furthermore, no putative ρ-independent terminators were detected in the yohC–pbpG intergenic region. Interestingly, this specific targeting of pbpG expression appears to correlate with the requirement of PBP 7 in the SSR.

A pbpG-specific transcript was detected in exponential-phase cells as the major transcript (Figs 3a, 4b). This transcript decreased significantly in 5 h and 24 h C-starved cells as the yohC–pbpG co-transcript increased (Figs 3a, 4b). Furthermore, this transcript was still present in the stiC2::MudJ mutant (Fig. 3a), indicating the presence of a pbpG-specific promoter (pbpGp). In ST68, pbpG transcript levels were similar in exponential-phase and 5 h C-starved cells.
cells (Fig. 3a), indicating that without CSI read-through transcription pbpG-specific transcription reaches constitutive levels. Fig. 3(b) not only shows that pbpG was not CSI, but also that it is not regulated by σS or cAMP-CRP.

Results presented above indicated the existence of at least two promoters within the yohC–pbpG operon, a cAMP-CRP-negatively regulated σS-dependent CSI yohCp and a constitutive pbpGp. The rpoS-dependency of yohC expression is also supported by separate studies in E. coli looking at the global gene expression during growth in glucose-limited continuous cultures (Franchini & Egli, 2006) and seawater (Rozen & Belkin, 2001). Both studies reported that yohC is induced and regulated by rpoS. Not surprisingly, the most important factor effecting yohC expression in seawater was nutrient deprivation (Rozen & Belkin, 2001).

Identification of TSPs for yohCp and pbpGp
To localize the positions of the yohCp and pbpGp promoters in exponential-phase and C-starved cells, high-resolution S1-nuclease mapping was performed using several 5’-labelled probes (Fig. 4a) and RNA isolated from exponential-phase, 5 h and 24 h C-starved SL1344. As shown in Fig. 4b (centre), several closely migrating RNA-protected fragments were identified using S1 probe 1 (corresponds to yohCp). The intensity of these fragments was greatest in 5 h C-starved cultures. S1 probe 3 was used to identify transcripts corresponding to pbpGp, and again a number of closely migrating RNA-protected fragments were identified. The intensity of these fragments was highest in exponential-phase cells and dramatically lowered in 5 h and 24 h C-starved cells (Fig. 4b, right). In addition, a longer RNA-protected fragment was identified with S1 probe 3, corresponding to...
the CSI yohCp-promoted transcript identified with S1 probe 1 (Fig. 4b, centre). Thus, pbpG expression was promoted by both the CSI yohCp and constitutive pbpGp promoters. Similarly, S1 probe 2 (Fig. 4a) produced several closely migrating RNA-protected fragments corresponding to yohDp. The intensity of these fragments was highest in exponential-phase cells and slightly lower in 5 h C-starved cells but undetected in 24 h C-starved cells (Fig. 4b, left). The intensities of the protected fragments detected under all three conditions corresponded well with our Northern hybridization and promoter–lac fusion results, presented above.

Although S1-nuclease mapping is more reliable for determining TSP, for some A/T-rich RNA:DNA hybrids S1-nuclease can sometimes ‘end-nibble’, resulting in two or more closely migrating RNA-protected fragments. This may obscure the precise location of TSP of the promoter(s) (Kormanec, 2001). Since this was a problem with all three promoters, we employed primer extension using the same 5'-labelled primers to more precisely localize the TSPs. Because protected fragments were detected in 5 h C-starved cells for all three promoters, RNA from 5 h C-starved cells was used. As shown in Fig. 4 (c), the precise position of each promoter was confirmed by the detection of a single primer extension product, corresponding to a single TSP for each gene.

TSP identification plus DNA sequence analysis revealed a sequence (5’-ATTATACTTGA-3’) closely matching (8 out of 11 match overall; 6 out of 8 match of most conserved) the consensus sequence for σ70 promoters (Lacour et al., 2003; Weber et al., 2005), located 35 bp upstream of the initiation codon for the YohC ORF. A putative −35 region (5’-TTCATA-3’) closely matching (4 out of 6 match) the consensus −35 sequence was located 18 bp upstream (Fig. 1). The presence of a −35 region for σ70 promoters was recently proposed, with −10/−35 spacing being more flexible. Although the yohCp spacer length of 18 bp is suboptimal, it does show A/T-richness (~73%), which is proposed to stimulate σ70 promoter activity (Typas & Hengge, 2006). A generally poor σ70 promoter (Moat et al., 2002) based on TSP localization for yohD was identified approximately 60 bp upstream of yohCp, indicating that the two promoters do not directly overlap (Fig. 1).

Based upon TSP localization, a potential σ70 promoter sequence (Moat et al., 2002) possessing a near-canonical −10 site (5’-TATGAT-3’; 5 out of 6 match) and a poor −35 site (5’-TAGGCG-3’; only 2 out of 6 match) with a 17 bp spacer, was detected 79 bp upstream from the pbpG initiation codon. Thus, pbpGp has a good −10 and spacer region but lacks a clear −35 site (Fig. 1). A potential σ5 promoter could also be discerned but pbpGp::lac analysis did not demonstrate any σ5 regulation (Fig. 3b).

**CSI levels of pbpG are required for maximal SSR development in 5 h C-starved Salmonella**

We previously reported that a stiC::MudJ insertion mutant exhibits a deficient SSR (Spector & Cubitt, 1992; O’Neal et al., 1994; Seymour et al., 1996). However, the revelation that the stiC insertion affects CSI pbpG expression, but not constitutive pbpG expression (Fig. 3a), provoked further study. To determine if stiC2::MudJ phenotypes resulted from a lack of yohC or polar effects on pbpG expression during C-starvation, a ΔyohC18 mutant (SMS923; yohC− pbpGconst.+/induc.+) that lacks yohC but still produced CSI levels of pbpG and a ΔyohC17::Ω-Km′ lacking both yohC and CSI levels of pbpG expression (SMS863; yohC− pbpGconst.+/induc.−) were tested. The expression profiles of both SMS863 and SMS923 were confirmed using RT-PCR (data not shown). Results presented in Fig. 5 showed that CSI pbpG levels, but not constitutive pbpG levels, were necessary to develop a

![Image](http://mic.sgmjournals.org)
yohC pbpG operon is induced intracellularly within cultured MDCK cells

S. Typhimurium is a facultative intracellular pathogen (Finlay & Falkow, 1989), and so it is important to know what functions are expressed within the host to provide valuable insights into the intracellular environment as well as potential roles in pathogenesis (Mahan et al., 1995; Valdivia & Falkow, 1997). To examine this, MDCK epithelial cells were infected with ST68 and stiC2::lac fusion (i.e. yohC pbpG) expression was monitored. Intracellular β-galactosidase activity expression was measured at 6 h post-infection by comparing activity in intracellular bacteria with extracellular bacteria. Results indicated that stiC2::lac was induced 12.5 ± 3.72-fold (mean ± SEM, n=4) within MDCK epithelial cells. This induction ratio was similar to the fold-induction determined in 5 h C-starved cells compared to exponential-phase cells. These results support a model that Salmonella are either C-starved or exposed to conditions generating overlapping signals inside MDCK epithelial cells and perhaps other cells. However, the intracellular induction of yohC pbpG did not translate into attenuation of virulence potential (LD50) in a BALB/c mouse virulence model; the LD50 (10^4.2) for ST68 was equivalent to the LD50 (10^4.5) for SL1344. This can mean that inducible levels of yohC and/or pbpG are not essential for virulence in this model, or that compensatory functions may be expressed that can mask the need for induced levels of these genes; similar to the differential phenotypic effects observed in 5 h and 24 h C-starved cells described above.

Conclusions

The expression and phenotypic data presented here all support a model whereby CSI expression of pbpG from the yohC promoter, but not constitutive expression of pbpG from its own pbpG promoter, is required for the SSR in 5 h C-starved cells, but not in 24 h C-starved cells. Our data also indicate that yohC is not required for SSR function in C-starved cells. Thus, induced levels of the DD-endopeptidase activity of PBP 7 appear to function within the first few hours of C-starvation, but later become expendable, possibly as new functions are expressed to overcome the deficiency, particularly in terms of development of cross-resistances.

The question is, how does a DD-endopeptidase activity contribute to CSI cross-resistance in C-starved cells, as well as to LT-CSS? The answer to this question is likely to be complicated, given the findings that E. coli cells lacking one or more combinations of PBPs are viable (Denome et al., 1999; Heidrich et al., 2002). The one relevant caveat to those studies is that they primarily looked at growing cells or stationary-phase cells grown in rich media. In E. coli, cell wall and cell shape changes occur early during stationary-phase in rich medium (Dougherty & Pucci, 1994; Meberg et al., 2004). Similar size and shape changes also occur in S. Typhimurium during C-starvation (M. Spector, unpublished observations). Tuomanen & Cozens (1987) showed that peptidoglycan composition changed as growth rates slowed (due essentially to C-source limitation in chemostat cultures), and cell volume decreased leading to smaller, more coccoid-shaped cells. They proposed that this is due to alterations in the level of activities of several PBPs; however, their study did not examine a role for PBP 7 activity. A proposed role for PBP 7 in cell-wall remodelling (Romeis & Höltje, 1994), daughter-cell separation (Heidrich et al., 2002; Priyadarshini et al., 2006) and cell morphology (Meberg et al., 2004) could help explain a role for PBP 7 in the reduction in cell volume and change in cell shape that occurs during the early stages of the SSR. PBP 7 and PBP 4 are endopeptidases that degrade the peptide cross-links between glycan chains in the cell wall (Romeis & Höltje, 1994). Meberg et al. (2004) proposed that cell shape may be governed by the presence and locations of specific types of peptide cross-links, with PBP 4 and 7 functioning to cleave so-called inappropriate cross-links. Tuomanen & Schwartz (1987) proposed a role for PBP 7 in inhibiting autolysis of non-growing E. coli by contributing to the production of autolysis-resistant peptidoglycan. This hypothesis is based on the profile of β-lactam antibiotics that bind to PBP 7 and their differential ability to lyse non-growing E. coli cells. It should be noted that in most of these studies, PBP 7’s role has been determined to be conditional (detectable only if some other function is missing) or minor. The C-starvation induction of pbpG and SSR-defective phenotype associated with PBP 7 levels in the 5 h C-starved cell may have deciphered a role for PBP 7 DD-endopeptidase activity in producing an appropriate peptidoglycan structure that is necessary for cell survival (or inhibition of autolysis) under certain conditions (e.g. exposure to high temperatures or oxidative damaging agents). The apparent dispensability of PBP 7 in 24 h C-starved cells may be due to expression of compensatory functions such as PBP 4 or MepA (Denome et al., 1999); these possibilities are currently under investigation.
C-starvation, since a reduction in cell size during the early stages of the SSR could: (i) allow, for example, membrane phospholipids to be used as a C-energy source early during the SSR and/or (ii) reduce the need for biosynthesis of phospholipids in the C-starved cell. The former is supported by our previous report that the key fatty acid degradation enzyme FadF (medium/long-chain fatty acyl-CoA dehydrogenase) is C-starvation-inducible and required for long-term C-starvation survival (Spector et al., 1999a).

In closing, the level and timing of PBP7 expression in S. Typhimurium is clearly important, since the bacteria appear to actively control the levels of pbpG-containing transcripts in cells during C-starvation (Figs 3, 4b). The reason for this presents an intriguing problem to solve.

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REFERENCES


inducible adaptive resistance to oxidative challenge in
Salmonella enterica serovar Typhimurium. Microbiology 152, 1347–1359.

nutrient-starvation conditions in Salmonella typhimurium. FEMS


Salmonella typhimurium: regulation and roles in starvation survival.
Mol Microbiol 6, 1467–1476.

(SSR) of Salmonella typhimurium: gene expression and survival
during nutrient starvation. In Starvation in Bacteria, pp. 201–224.

Global control in Salmonella typhimurium: two-dimensional gel
electrophoretic analysis of starvation-, anaerobiosis-, and heat-shock-

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

Spector, M. P., DiRusso, C. C., Pallen, M. J., Garcia del Portillo, F.,
acyl-CoA dehydrogenase (fadF) gene of Salmonella typhimurium is
a phase 1 starvation-stress response (SSR) locus. Microbiology 145,

Spector, M. P., Garcia del Portillo, F., Bearson, S. M., Mahmud, A.,
Magut, M., Finlay, B. B., Dougan, G., Foster, J. W. & Pallen, M. J.
(1999b). The rpoS-dependent starvation-stress response locus
stlA encodes a nitrate reductase (narZWV) required for carbon-
starvation-inducible thermotolerance and acid tolerance in
Salmonella typhimurium. Microbiology 145, 3035–3045.

Tanaka, K., Takayanagi, Y., Fujita, N., Ishihama, A. & Takahashi, H.
(1993). Heterogeneity of the principal r factor in Escherichia coli: the
rpoS gene product, rpoS24, is a second principal r factor of RNA
polymerase in stationary-phase Escherichia coli. Proc Natl Acad Sci
U S A 90, 3511–3515.

Testerman, T. L., Vaizez-Torres, A., Xu, Y., Jones-Carson, J., Libby,
S. J. & Fang, F. C. (2002). The alternative sigma factor r controls
antioxidant defenses required for Salmonella virulence and stationary-

composition and peptidoglycan-binding proteins in slowly growing

its relationship to lysis of non-growing Escherichia coli. J Bacteriol
169, 4912–4915.

Tyapas, A. & Hengge, R. (2006). Role of the spacer between the rpoS
and rpoC2 regions on rpoS promoter selectivity in Escherichia coli.
Mol Microbiol 59, 1037–1051.


Weber, H., Polen, T., Heuveling, J., Wendisch, V. F. & Hengge, R.
in Escherichia coli: rpoS-dependent genes, promoters, and sigma factor

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