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σ^S-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, σ^S-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 σ^S–dependent C-starvation-inducible promoter upstream of *yohC*. Another (σ^S-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.

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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

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Abbreviations: CSI, carbon-starvation-inducible; LT-CSS, long-term carbon-source starvation survival; PBP, penicillin-binding protein; SP-PCR, single-primer PCR; SSR, starvation-stress response; TSP, transcriptional start point.

Alignments of YohC proteins and phylogenetic tree are available with the online version of this paper.

(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^{24}) and *rpoD* (σ^D or σ^{70}) (Jenkins *et al.*, 1988; McCann *et al.*, 1991; Fang *et al.*, 1992; Spector & Cubitt, 1992; Tanaka *et al.*, 1993; Loewen & Hengge-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, AmiA, AmiB, and AmiC, 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 (Neidhardt *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

Strains, plasmids and primers	Relevant genotype or phenotype information*	Reference or source
Bacterial strains		
SL1344	*hisG46* (mouse-virulent *Salmonella enterica* serovar Typhimurium)	Hoiseth & Stocker, (1981)
ST68	SL1344 *stiC1*::Mu*dJ* (*lac* Km^r)	Spector & Cubitt, (1992)
LB5000	LT-2 i*lv452 metA22 metE551 trpD2 leu hsdLT hsdSA hsdSB*	Bullas & Ryu, (1983)
SMS758	SL1344/pRS1274 (Ap^r)	This work
SMS818	SL1344/pKS25 (Ap^r)	This work
SMS819	SL1344/pKS26 (Ap^r)	This work
SMS827	SL1344 *rpoS24*::Tn*10d* (Tc^r) / pRS1274 (Ap^r)	This work
SMS828	SL1344 *rpoS24*::Tn*10d* (Tc^r) / pKS17 (Ap^r)	This work
SMS829	SL1344 *rpoS24*::Tn*10d* (Tc^r) / pKS25 (Ap^r)	This work
SMS830	SL1344 *rpoS24*::Tn*10d* (Tc^r) / pKS26 (Ap^r)	This work
SMS831	SL1344 *crp-773*::Tn*10* (Tc^r) / pRS1274 (Ap^r)	This work
SMS832	SL1344 *crp-773*::Tn*10* (Tc^r) / pKS17 (Ap^r)	This work
SMS833	SL1344 *crp-773*::Tn*10* (Tc^r) / pKS25 (Ap^r)	This work
SMS834	SL1344 *crp-773*::Tn*10* (Tc^r) / pKS26 (Ap^r)	This work
SMS863	SL1344 Δ*yohC17*::Ω-Km^r	This work
SMS923	SL1344 Δ*yohC18* (Km^s; SMS863 with Ω-Km^r cassette removed)	This work
EC33	*Escherichia coli* K-12 (λ^S) (Stanford strain)	Jenkins *et al.*, (1988)
MC4100	*araD159* Δ[*argF-lac*]*U169 rpsL150 relA1 ftb5301 deoC1 ptsF25 rbsR*	Bébien *et al.*, (2002)
Plasmids		
pKD4	Vector carrying the FRT-Ω-Km^r-FRT cassette (Ap^r)	Datsenko & Wanner, (2000)
pKD46	Ts (30 °C) replicon; encodes arabinose-inducible β, γ and Exo proteins of λ-Red recombination system (Ap^r)	Datsenko & Wanner, (2000)
pCP20	Ts (30 °C) replicon carrying Flp recombinase gene (Ap^r Cm^r)	Datsenko & Wanner, (2000)
pRS1274	Transcriptional (promoter-less) *lac* fusion vector (Ap^r)	Simons *et al.*, (1987)
pKS17	*yohD-yohC* intergenic region in pRS1274 (*yohD*p orientation); Ap^r	This work
pKS25	*yohD-yohC* intergenic region in pRS1274 (*yohC*p orientation); Ap^r	This work
pKS26	*yohC-pbpG* intergenic region in pRS1274 (*pbpG*p orientation); Ap^r	This work
Primer	**Oligonucleotide sequence**	
PR2	5′-AGTGGGCATAGCCATACCAG-3′	
PR7	5′-GCATCGTCCTAACAGATAAAGTAGC-3′	
PR53	5′-ATACGCACCATGTTTTGCTG-3′	
PR56	5′-GGCATGACTTTACCGGTTTC-3′	
PR92	5′-GTAAACCACCTCCGGCATTA-3′	
PR93	5′-ATACGGCTACCGCCTGTTCT-3′	
PR94	5′-TCAGGAATGAAGCTCGGAAT-3′	
PR95	5′-AAAAGCCCCCATACATGGTT-3′	
PR118	5′-ATGAACCATGTATGGGGGCTTTTTTCCCATCCCGATCGGGGTGTAGCTG-GAGCTGCTTC-3′	
PR119	5′-CCCCACAGGATCACGGTGATCGCCAGTAGCACCTCCAGGACATATGAAT-ATCCTCCTTA-3′	
yohCREV	5′-CGCAGACGACGGGAATCGCC-3′	
yohCFOR	5′-GCCACCAGCGGAAACTTGAGC-3′	
pbpGREV	5′-CCGTTTTCGCCACAGCCTGCG-3′	

*Km^r, Kanamycin resistance; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Sm^r, streptomycin resistance; Cm^r, chloramphenicol resistance.

by dividing the c.f.u. ml^{-1} at each time point by the c.f.u. ml^{-1} 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 *Sma*I 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* (*yohD*p; pKS17) and *yohC* (*yohC*p; pKS25). The *yohC–pbpG* intergenic region was similarly amplified and cloned in the *pbpG* promoter orientation (*pbpG*p; pKS26).

β-Galactosidase assay. Desired strains were grown and starved as described above. At the appropriate times, cells were assayed for β-galactosidase activity. β-Galactosidase activity was expressed in Miller units (Miller, 1992). Data presented are means ± SEM for at least three separate trials.

Selenate/selenite 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^r) insertion site in the chromosome. The insertion site for the *stiC2*::MudJ (*lac* Km^r) 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 (Δ*yohC18*::Ω-Km^r) was accomplished using a modified λ-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 Δ*yohC17*::Ω-Km^r mutation was then transduced into SL1344 to generate SMS863.

Flp recombinase protocol. The Ω-Km^r cassette was removed from SMS863 chromosome using Flp recombinase as described by Datsenko & Wanner (2000). pCP20 (carries Flp recombinase gene) was electroporated into freshly prepared electrocompetent SMS863 cells using an *E. coli* Pulser (Bio-Rad) and recovered on LB Ap plates at 30 °C. Transformant colonies were streaked onto multiple LB plates and incubated overnight at 30 °C and 43 °C. Colonies growing at 43 °C were scored onto LB, LB Ap and LB Km plates and incubated at 37 °C for 18–24 h. The subsequent Ap- and Km-sensitive strains were screened for the loss of the Ω-Km^r cassette and presence of the Δ*yohC18* mutation using PCR. The subsequent strain SMS923 was stored at −80 °C.

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 3′-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 *yohC*p) was a 421 bp DNA fragment prepared by PCR using a 5′-labelled yohCREV primer and unlabelled yohCFOR primer; (ii) S1 probe 2 (for *yohD*p) 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 *pbpG*p) 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 [γ-^{32}P]ATP (1.665×10^{14} 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 β-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^r) insertion lies in the *yohC* homologue of *S. enterica*

We previously reported that a cAMP-CRP-negatively regulated σ^S-dependent CSI gene locus designated *stiC*

was required for the SSR in *S.* Typhimurium (Spector *et al.*, 1988; Spector & Cubitt, 1992; O'Neal et al., 1994; Seymour *et al.*, 1996). We analysed the DNA adjacent to a *stiC2*::MudJ 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 ($YohC_{STM}$) 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). $YohC_{STM}$ 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 $YohC_{STM}$. The N-terminal domain of $YohC_{STM}$, and its homologues, possesses multiple conserved histidine residues (six in $YohC_{STM}$), suggesting that this motif is involved in the function of $YohC_{STM}$ and its homologues.

Bébien *et al.* (2002) reported that an *E. coli yohC* mutant is unable to reduce selenate to elemental selenium, but is able to reduce selenite to selenium, suggesting it is defective in selenate transport. We screened our Δ*yohC17*::Ω-Km^r (SMS863) and Δ*yohC18* (SMS923) mutants and their SL1344 parent for the ability to reduce selenite or selenate to elemental selenium. All were able to reduce selenite and selenate to elemental selenium (data not shown). The reason for the discrepancies between Bébien *et al.* (2002) and our results are not known. Two possibilities are: (i) $YohC_{STM}$ function has diverged from $YohC_{ECO}$, possibly with respect to substrate specificity; or (ii) *S.* Typhimurium possesses 1 or more additional transport systems that can compensate for the loss of YohC. The first scenario is supported phylogenetically. The YohC homologues of four *Salmonella* serovars showed significant divergence from the YohC homologues of several *E. coli* and *Shigella* species (see Supplementary Figure S2 for phylogenetic data, available in the online version of the paper). Nonetheless, $YohC_{STM}$ is not required for selenate transport under our conditions. However, we cannot rule out a role in selenate transport [scenario (ii) above].

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 PBP 7. 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 upstream of *yohC*.

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 PBP 8 from PBP 7 (Henderson *et al.*, 1994) in *E. coli*. Thus, STM2168 seems to encode the *S.* Typhimurium PBP 7.

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

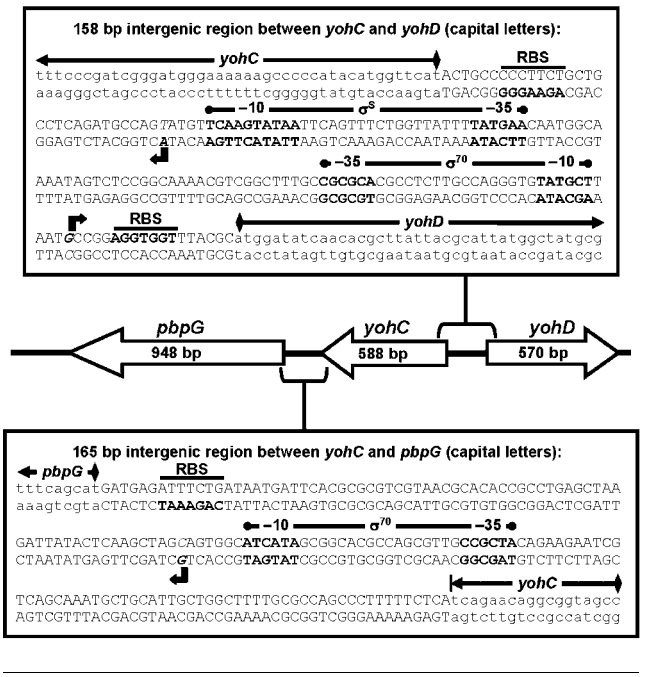

Fig. 1. Schematic illustrating the organization of the *yohD yohC pbpG* region of the *Salmonella enterica* chromosome. Predicted σ^{70}-dependent promoters for *yohD* and *pbpG* and σ^{S}-dependent promoter for the *yohC pbpG* operon are shown. ORFs are indicated by lower-case letters. Predicted ribosome-binding sites (RBS) are designated. The TSP for the individual genes is indicated by thick bent arrows.

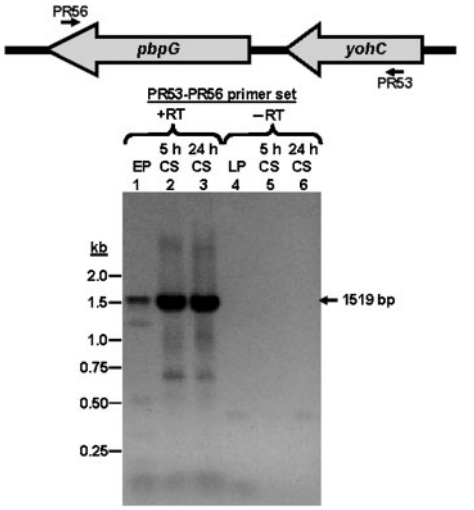

Fig. 2. RT-PCR products indicating the presence of a bicistronic transcript covering both the *yohC* and *pbpG* ORFs. Primers specific for *yohC* (PR53) and *pbpG* (PR56) were used to amplify RNA from exponential-phase (EP), 5 h C-starved (5 h CS) and 24 h C-starved (24 h CS) cells. Reaction mixtures contained *Taq* polymerase with (+RT) or without (−RT) reverse transcriptase. Results are representative of at least three separate experiments.

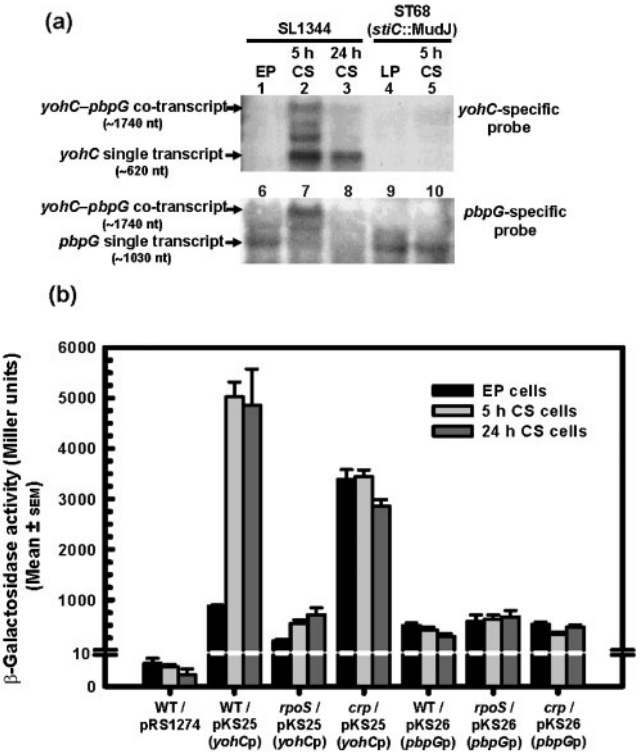

Fig. 3. Northern blot and promoter–*lacZ* fusion analyses of the *yohC pbpG* operon. (a) RNA from exponential-phase (EP), 5 h C-starved (5 h CS) and 24 h C-starved (24 h CS) SL1344 and ST68 (*stiC2*::MudJ) cells was probed with *yohC*- or *pbpG*-specific probes. (b) Exponential-phase (EP cells), 5 h (5 h CS cells) and 24 h (24 h CS cells) C-starved wild-type, *rpoS24*::Tn*10d* (Tc^r) and *crp-773*::Tn*10* (Tc^r) mutant cells carrying pKS25 (*yohC*p) or pKS26 (*pbpG*p) were analysed for β-galactosidase expression (Miller, 1992). Northern blot analyses were performed two times with independent RNA preparations with similar results. Promoter–*lac* fusion data represent the mean ± SEM of at least three separate experiments.

C-starved cells compared to exponential-phase cells. This is supported by Northern hybridization and TSP analyses.

The *yohC*p, but not *pbpG*p, 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 (*yohC*p) 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^r) 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 *yohC*p::*lacZ* (Fig. 3b) analyses in 24 h C-starved cells showed that the *yohC*p 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 (*pbpG*p). In ST68, *pbpG* transcript levels were similar in exponential-phase and 5 h C-starved

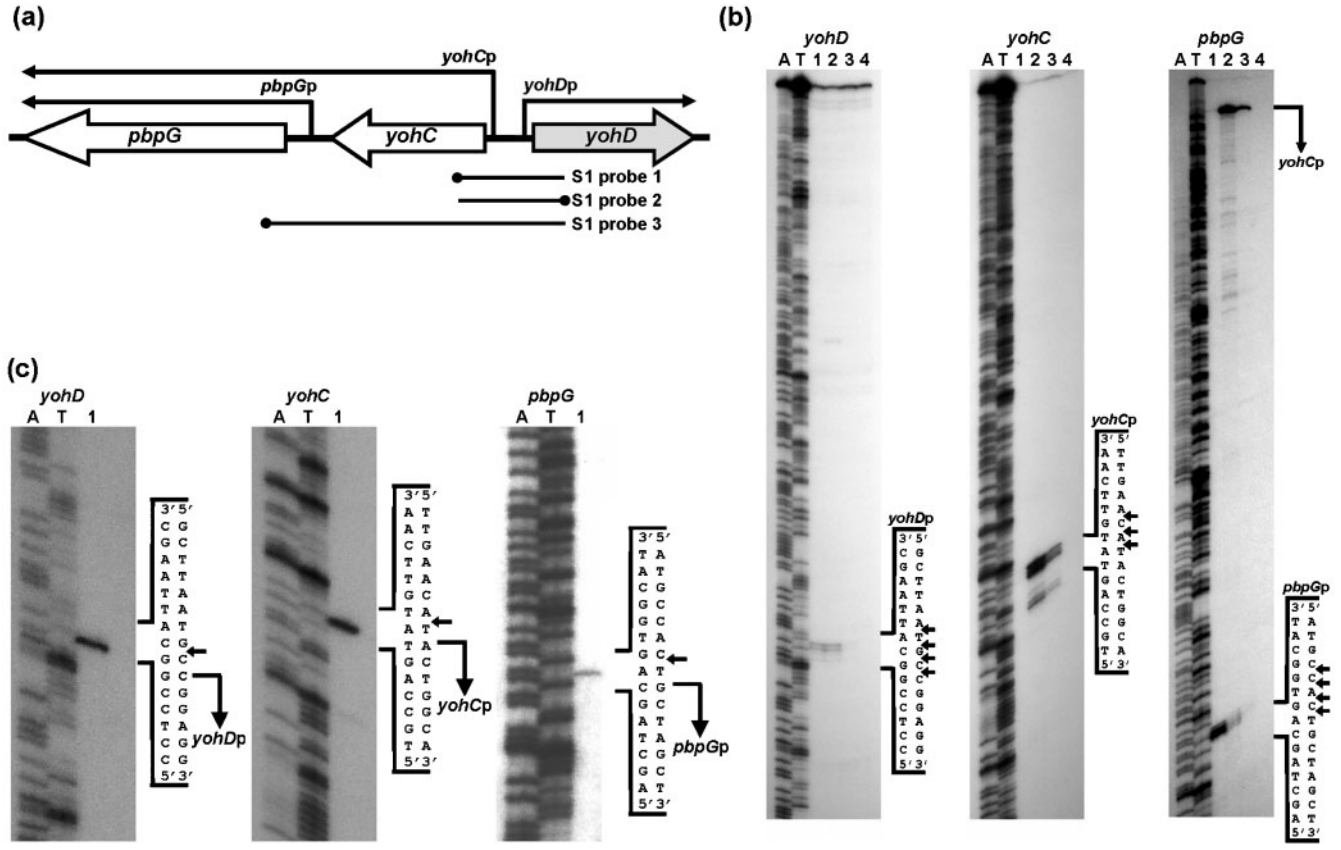

Fig. 4. TSP determination for the *yohD*, *yohC* and *pbpG* genes. (a) Schematic showing the probes used for S1-nuclease mapping. The lines below the map represent 5′-labelled (closed circle) DNA fragments used as probes. S1 probe 1, *yohC*; S1 probe 2, *yohD* and S1 probe 3, *pbpG*. (b) High-resolution S1-nuclease mapping of the TSPs for *yohD*p, *yohC*p and *pbpG*p. RNA probed from exponential-phase (lane 1), 5 h C-starved (lane 2) and 24 h C-starved (lane 3) SL1344. Lane 4 represents *E. coli* tRNA as a control. Protected DNA fragments were analysed on DNA sequencing gels with G+A (lane A) and T+C (lane T) sequencing ladders (Maxam & Gilbert, 1980). Arrows indicate RNA-protected fragments. (c) Primer extension analysis of the TSPs for *yohD*p, *yohC*p and *pbpG*p using RNA from 5 h C-starved SL1344. The primer extension products (lane 1 in each panel) were analysed on DNA sequencing gels with G+A (lanes A) and T+C (lanes T) sequencing ladders. Arrows indicate RNA-protected fragments. Bent arrows indicate TSP for indicated promoter. All S1-nuclease mapping and primer extension experiments were performed twice using independent sets of RNA with similar results.

cells (Fig. 3a), indicating that without CSI read-through transcription *pbpG*-specific transcription reaches constitutive levels. Fig. 3(b) not only shows that *pbpG*p was not CSI, but also that it is not regulated by σ^S or cAMP-CRP.

Results presented above indicated the existence of at least two promoters within the *yohC–pbpG* operon, a cAMP-CRP-negatively regulated σ^S-dependent CSI *yohC*p and a constitutive *pbpG*p. 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 *yohC*p and *pbpG*p

To localize the positions of the *yohC*p and *pbpG*p 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. 4 (b) (centre), several closely migrating RNA-protected fragments were identified using S1 probe 1 (corresponds to *yohC*p). The intensity of these fragments was greatest in 5 h C-starved cultures. S1 probe 3 was used to identify transcripts corresponding to *pbpG*p, 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 *yohC*p-promoted transcript identified with S1 probe 1 (Fig. 4b, centre). Thus, *pbpG* expression was promoted by both the CSI *yohC*p and constitutive *pbpG*p promoters. Similarly, S1 probe 2 (Fig. 4a) produced several closely migrating RNA-protected fragments corresponding to *yohD*p. 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 σ^S 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 σ^S promoters was recently proposed, with −10/−35 spacing being more flexible. Although the *yohC*p spacer length of 18 bp is suboptimal, it does show A/T-richness (~73 %), which is proposed to stimulate σ^S promoter activity (Typas & Hengge, 2006). A generally poor σ^{70} promoter (Moat *et al.*, 2002) based on TSP localization for *yohD* was identified approximately 60 bp upstream of *yohC*p, indicating that the two promoters do not directly overlap (Fig. 1).

Based upon TSP localization, a potential σ^{70} 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′-TAGCGG-3′; only 2 out of 6 match) with a 17 bp spacer, was detected 79 bp upstream from the *pbpG* initiation codon. Thus, *pbpG*p has a good −10 and spacer region but lacks a clear −35 site (Fig. 1). A potential σ^S promoter could also be discerned but *pbpG*p : : *lac* analysis did not demonstrate any σ^S regulation (Fig. 3b).

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

We previously reported that a *stiC2* : : 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 Δ*yohC18* mutant (SMS923; *yohC*$^-$ *pbpG*$^{const.+/induc.+}$) that lacks *yohC* but still produced CSI levels of *pbpG* and a Δ*yohC17* : : Ω-Km^r lacking both *yohC* and CSI levels of *pbpG* expression (SMS863; *yohC*$^-$ *pbpG*$^{const.+/induc.-}$) 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

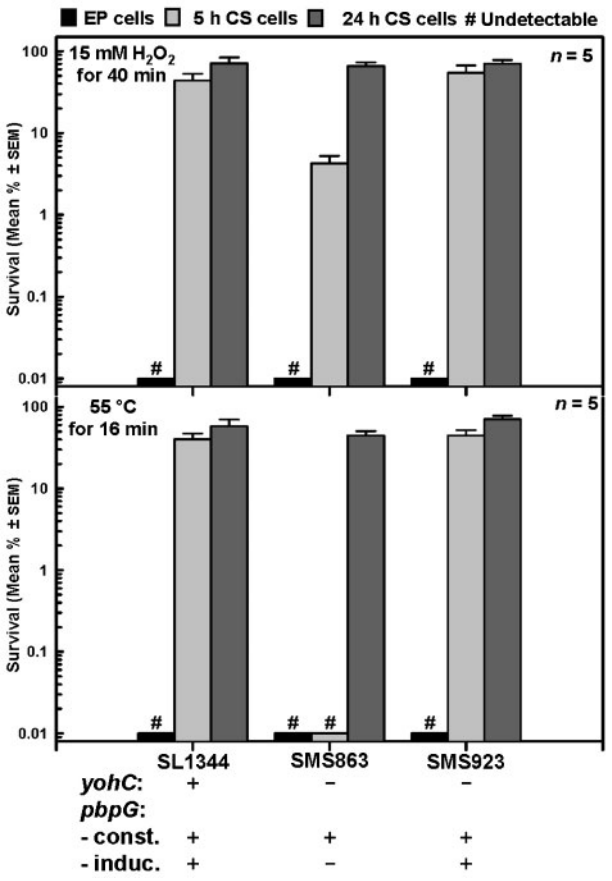

Fig. 5. Inducible levels of *pbpG* expression are required for CSI cross-resistance to oxidative and thermal stress. Strains SL1344 (parent), SMS863 (Δ*yohC17* : : Km^r) and SMS923 (Δ*yohC18*) were grown to exponential-phase (EP cells) or C-starved for 5 h (5 h CS cells) or 24 h (24 h CS cells) before being challenged with the indicated stress. The status of *yohC* and *pbpG* expression is shown; for *pbpG*, const., constitutive expression from *pbpG*p and induc., inducible expression from CSI *yohC*p. A (+) and (–) sign indicates the presence or absence of that phenotype, respectively. 100 % survival was typically ca 3–5×10^6 c.f.u. ml^{-1}. The number (*n*) of independent experiments for each is indicated.

maximal SSR in 5 h C-starved cells, but not in 24 h C-starved cells. Five h C-starved, but not 24 h C-starved SMS863 cells, were defective in CSI cross-resistance to a 40 min challenge with 15 mM H_2O_2 and a 16 min challenge at 55 °C, compared to SL1344 and SMS923 cells. The defect was particularly significant in CSI cross-resistance to 55 °C (Fig. 5). Additionally, SMS863, but not SMS923, showed reduced LT-CSS (data not shown). However, neither SMS863 nor SMS923 were defective in CSI cross-resistance to a 60 min challenge at pH 3.0 (data not shown). These phenotypic patterns correlated well with *pbpG* expression patterns in 5 h C-starved cells, compared to 24 h C-starved cells (Figs 3a, b and 4b). This is the first report of a detectable phenotype associated with *pbpG* expression independent of the expression of other PBPs.

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 $\pm$ SEM, $n=4$) within MDCK epithelial cells. This induction ratio was similar to the fold-induction determined in 3 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 (LD_{50}) in a BALB/c mouse virulence model; the LD_{50} ($10^{4.2}$) for ST68 was equivalent to the LD_{50} ($10^{4.5}$) 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*p promoter, but not constitutive expression of *pbpG* from its own *pbpG*p 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 very 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.

PBP 7's potential role in cell-shape determination might also be beneficial for the long-term survival of cells during

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 PBP 7 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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