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Shifts from glucose to certain secondary carbon-sources result in activation of the extracytoplasmic function sigma factor σ^E in *Salmonella enterica* serovar Typhimurium

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Abstract

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) elicits the starvation-stress response (SSR) due to starvation for an essential nutrient, e.g. a carbon/energy source (C-source). As part of the SSR, the alternative sigma factor σ^E is activated and induced. The authors suspect that this activation is, in part, triggered by changes in the *S. Typhimurium* cell envelope occurring during the adaptation from growth to carbon/energy starvation (C-starvation), and resulting in an increased need for σ^E -regulated factors involved in the proper folding and assembly of newly synthesized proteins destined for this extracytoplasmic compartment. This led to the hypothesis that a σ^E activation signal might arise during C-source shifts that cause the induction of proteins localized to the extracytoplasmic compartment, i.e. the outer membrane or periplasm, of the cell. To test this hypothesis, cultures were grown in minimal medium containing enough glucose to reach mid-exponential-phase, plus a non-limiting amount of a secondary 'less-preferred' but utilizable carbon/energy source. The σ^E activity was then monitored using plasmids carrying *rpoEP1*– and *rpoEP2*–*lacZ* transcriptional fusions, which exhibit σ^E -independent and -dependent *lacZ* expression, respectively. The secondary C-sources maltose, succinate and citrate, which have extracytoplasmic components involved in their utilization (e.g. LamB), resulted in a discernible diauxic lag period and a sustained increase in σ^E activity. Growth transition from glucose to other utilizable phosphotransferase (PTS) and non-PTS C-sources, such as trehalose, mannose, mannitol, fructose, glycerol, D-galactose or L-arabinose, did not cause a discernible diauxic lag period or a sustained increase in σ^E activity. Interestingly, a shift from glucose to melibiose, which does not use an extracytoplasmic-localized protein for uptake, did cause an observable diauxic lag period but did not result in a sustained increase in σ^E activity. In addition, overexpression of LamB from an arabinose-inducible promoter leads to a significant increase in σ^E activity in the absence of a glucose to maltose shift or C-starvation. Furthermore, a *lamB* : Ω -Km^r mutant, lacking the LamB maltoporin, exhibited an approximately twofold reduction in the sustained σ^E activity observed during a glucose to maltose shift, again supporting the hypothesis. Interestingly, the LamB protein lacks the typical Y-X-F terminal tripeptide of the OmpC-like peptides that activate DegS protease activity leading to σ^E activation. It does, however, possess a terminal pentapeptide

(Q-M-E-I-W-W) that may function as a ligand for a putative class II PDZ-binding site. The authors therefore propose that the σ^E regulon of *S. Typhimurium* not only is induced in response to deleterious environmental conditions, but also plays a role in the adaptation of cells to new growth conditions that necessitate changes in the extracytoplasmic compartment of the cell, which may involve alternative signal recognition and activation pathways that are independent of DegS.

Abbreviations

C-source, carbon/energy source; C-starvation, carbon/energy starvation; IM, inner membrane; MS hiC, MOPS-buffered salts medium non-limiting in glucose; MS loC, MOPS-buffered salts medium limiting for glucose; OM, outer membrane; OMP, outer-membrane protein; PEP, phosphoenolpyruvate; PP, periplasmic; PTS, phosphotransferase; SSR, starvation-stress response

INTRODUCTION

Salmonella enterica serovar Typhimurium (*Salmonella* Typhimurium) is an aetiological agent of water/food-borne gastrointestinal-based disease in both animals and humans (Spector, 1998). An important feature of *S. Typhimurium* epidemiology and pathogenesis in both warm- and cold-blooded hosts is its ability to sense, adapt to and survive in a number of environments that expose the bacteria to stresses, such as nutrient starvation, extremes in osmolarity, temperatures or pH, which can threaten viability and/or limit growth (Spector, 1998; Humphreys *et al.*, 1999, 2003). Thus, bacterial responses to these stresses can affect the spread and pathogenesis of this important pathogen.

One of the most common stresses encountered by *S. Typhimurium*, and many other bacteria, outside the confines of the laboratory is starvation for a carbon/energy source (C-source). *S. Typhimurium*, as well as other non-spore-forming bacteria, responds to carbon/energy starvation (C-starvation) with global alterations in gene expression, morphology and physiology. This response is known as the starvation-stress response (SSR) (Spector & Cubitt, 1992; Seymour *et al.*, 1996; Spector, 1998; Spector *et al.*, 1999b). The SSR serves two functions: (1) to combat the long-term effects of C-starvation, and (2) to generate cross-resistance to other environmental stresses. The SSR includes aspects of: (a) avoidance, involving the production of new or higher-affinity C-source-utilization systems in an attempt to scavenge any utilizable C-sources from the environment; and (b) starvation/stress survival if an alternative C-source remains unavailable, ultimately generating a more efficient and hardy cell (Spector, 1998).

The SSR genes identified thus far are under the control of one or more of three sigma factors, σ^{70} , σ^S and σ^E (O'Neal *et al.*, 1994; Spector *et al.*, 1999a; Kenyon *et al.*, 2002). The alternative sigma factor σ^S is encoded by the *rpoS* gene (Tanaka *et al.*, 1993). During the SSR, σ^S is induced early in the response to C-starvation in both *Salmonella* and *Escherichia coli* (W. J. Kenyon & M. P. Spector, unpublished data; Fang *et al.*, 1992; Tanaka *et al.*, 1993), and during the shift from glucose to lactose utilization in *E. coli* (Fischer *et al.*, 1998). Glucose is the carbon source of choice for *Salmonella* and many other bacteria. While *Salmonella* is growing on glucose, the uptake and utilization of other carbon sources is inhibited, in part as a result of catabolite repression and/or inducer exclusion (Notley &

Ferenci, 1995; Moat *et al.*, 2002). Glucose exhaustion results in a shift to utilize alternative C-sources present in the growth medium. In *E. coli*, a shift from glucose to lactose utilization causes growth to transiently halt, producing what is called a diauxic lag period. During diauxie, functions needed for lactose utilization are induced, allowing the cell to resume growth. In addition, σ^S and σ^S -dependent genes are induced during this diauxic lag period (Fischer *et al.*, 1998).

The alternative sigma factor σ^E (encoded by the *rpoE* gene) is activated by certain stresses to the cell envelope that lead to the accumulation of denatured/unfolded proteins (Mecbas *et al.*, 1993, 1995; Missiakas *et al.*, 1996; Jones *et al.*, 1997; Alba *et al.*, 2002). Previous studies indicate that, once activated, σ^E initiates the transcription of several genes with extracytoplasmic functions required for the cell envelope to combat the effects of the stress. In the absence of stress, σ^E is normally bound to the inner-membrane (IM) protein RseA, an anti-sigma factor or inhibitor of σ^E , preventing its interaction with RNA polymerase, and thus the transcription of σ^E -dependent genes (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997; Alba *et al.*, 2002; Miticka *et al.*, 2003). When a specific stress occurs to the cell envelope, RseA is degraded by DegS and YaeL, σ^E is then released and is able to bind to core RNA polymerase (E). σ^E then directs the transcription of its dependent genes, many of which possess extracytoplasmic functions. A number of σ^E -regulated genes identified in *E. coli* encode cell-envelope-associated proteins that act on misfolded proteins, leading to their degradation, proper refolding or correct insertion into the outer membrane (OM), e.g. periplasmic (PP) proteases, peptidyl-prolyl isomerases and molecular chaperones (Erickson & Gross, 1989; Danese & Silhavy, 1997; Dartigalongue *et al.*, 2001; Alba *et al.*, 2002).

Previous findings in our laboratory (Kenyon *et al.*, 2002) showed that σ^E activity and protein levels increase during the initial 4–5 h of C-starvation, and reach peak levels at around 48–72 h of C-starvation. This suggests that C-starvation produces signals that lead to σ^E activation. Furthermore, *rpoE* mutants are defective in long-term C-starvation and C-starvation-inducible cross-resistance (Kenyon *et al.*, 2002), indicating that σ^E is an additional key regulator of the SSR. What is more, σ^E is critical for the virulence of *S. Typhimurium* in the mouse virulence model (Humphreys *et al.*, 1999).

In an effort to discern the role of the σ^E regulon in the SSR, we examined its activity in the avoidance component of the SSR by measuring σ^E -dependent transcription during shifts from glucose to less-preferred but utilizable C-sources. Here we report that shifts to some but not all secondary C-sources tested resulted in a sustained increase in σ^E activity. The common feature of C-sources resulting in increased σ^E activity appears to be the induction of an OM-associated and/or PP-binding-protein component involved in its utilization. Based on our findings and apparent common features of these utilization systems, we hypothesized that one or more members of the σ^E regulon is needed for the adaptation of the cell envelope to growth on less-preferred but utilizable C-sources, specifically those C-sources with transport systems possessing extracytoplasmic solute receptors and/or OM proteins. Therefore, σ^E -regulated functions appear to play a role not only in the responses to certain environmental stresses, but also in the adaptation of *Salmonella* to new environmental conditions not typically associated with cell envelope stress.

METHODS

Bacterial strains, plasmids and oligonucleotide primers—The bacterial strains, plasmids and oligonucleotide primers (Invitrogen) used are listed in Table 1. Transductions were carried out using the high-transducing derivative of bacteriophage P22, P22 HT *int-105* (Chan *et al.*, 1972). All strains were determined to be non-lysogens for P22 by demonstrating sensitivity to the H5 derivative of bacteriophage P22 (Maloy, 1990).

Media and supplements—Cultures of the desired strains were grown in a modified MOPS-buffered salts (MS) minimal medium (Neidhardt *et al.*, 1974) containing either 0.4 % (w/v) glucose (MS hiC) or 0.03 % (w/v) glucose (MS loC), and are described in detail elsewhere (Spector & Cubitt, 1992; O'Neal *et al.*, 1994). Histidine was added to minimal medium to a final concentration of 20 μ M. The appropriate antibiotics were added as needed: ampicillin (Ap) (30 μ g ml⁻¹), kanamycin (Km) (50 μ g ml⁻¹) and chloramphenicol (Cm) (50 μ g ml⁻¹). The rich medium used was Luria–Bertani (LB) broth and agar (Difco).

Identification of a *lamB* : : MudJ (*lac* Km^r) -carrying strain from a library of C-starvation-inducible MudJ : : *lac* fusion (insertion) strains—A *lamB* : : MudJ (*lac* Km^r) fusion-carrying strain was identified from our library of strains carrying MudJ (*lac* Km^r) insertions demonstrating C-starvation induction (Spector *et al.*, 1988, 1999a), using single-primer PCR, DNA sequence analysis and BLASTP search analysis as previously described (Spector *et al.*, 1999a, b). The insertion of the MudJ : : *lac* in *lamB* was confirmed by the presence of the correct size PCR product using chromosomal DNA from ST55 as a template, a forward primer specific for the 3'-end of MudJ (PR18) and a reverse primer specific for the 3'-end of *lamB* (PR49).

Growth and σ^E activation during C-source shifts—For growth and σ^E -activation analysis during glucose to alternative C-source shifts, overnight cultures of the desired strains were diluted 1 : 100 into fresh MS loC medium supplemented with one of the following: maltose, succinate, citrate, melibiose, D-trehalose, D-mannose, D-mannitol, D-fructose, glycerol, L-arabinose or D-galactose, all at a final concentration of 0.4 % (w/v), or no additional C-source (all C-sources were purchased from Sigma). The appropriate antibiotics were added as needed. The concentration of glucose in MS loC is the concentration used to produce C-starved cells (Spector & Cubitt, 1992; O'Neal *et al.*, 1994; Kenyon *et al.*, 2002) and provides sufficient glucose to support growth for 3–4 generations before being exhausted. Therefore, growth can only continue if an alternative utilizable C-source is available in the growth medium. Growth was monitored spectrophotometrically by determining OD₆₀₀ at pre-determined time intervals. Activation of σ^E was determined by measuring β -galactosidase activity as a reporter of promoter activity from plasmid pTF-P2; in addition, β -galactosidase activity driven by a *rpoE*-independent promoter (pTF-P1) was measured as a control (Kenyon *et al.*, 2002). β -Galactosidase activity was assayed by the method of Miller (1992) and is expressed in Miller units. The results presented represent the mean $\pm$ SEM from at least three separate experiments.

Generation of the *lamB* deletion mutant—The λ -Red mutagenesis system provides a more efficient way of disrupting and deleting chromosomal genes, allowing the characterization of a gene's function (Datsenko & Wanner, 2000), using the precise insertion of a selected antibiotic-resistance cassette into the gene of interest accompanied by a selected deletion. Using a slightly modified protocol and *S. Typhimurium* strain ST276 (carrying pKD46, a plasmid with the λ -Red recombination enzymes under the control of the L-arabinose-inducible P_{araBAD} promoter; Table 1), we constructed a defined deletion of the *lamB* gene from the *S. Typhimurium* chromosome, replacing it with an Ω -Km^r cassette.

Following the protocol of Datsenko & Wanner (2000), a PCR product was generated containing the Km^r cassette from pKD4 and sequence from the flanking region of the *lamB* gene of *S. Typhimurium*. PCR primers PR122 and PR123 (Table 1) were designed to contain a 40 bp sequence homologous to *lamB* DNA, and a sequence corresponding to the P1 and P2 sites on pKD4. PCR reactions using these primers, PCR SuperMix High Fidelity (Invitrogen) and pKD4 as a template were run in an Ericomp Thermal Cycler. The size of the PCR product (approximately 1.6 kb) was confirmed by agarose gel electrophoresis, and the DNA was cleaned using the Promega Wizard PCR Prep Kit. The DNA was then digested with *DpnI* (Promega) for 3 h at 37 °C, and cleaned using the Promega Wizard DNA Clean-up Kit.

Electrocompetent cells of strain ST276 were prepared by growing an overnight culture of ST276 in 1 ml LB-Ap with shaking at 37 °C. The following morning, the overnight culture was diluted 1 : 100 into 25 ml of LB containing 1 mM L-arabinose, and grown to an OD₆₀₀ of between 0.6 and 0.8. Freshly grown cells were pelleted, washed twice with 10 ml ice-cold sterile distilled water, and once with 10 ml ice-cold sterile 10 % (v/v) glycerol.

DpnI-digested DNA was then electroporated into the freshly prepared electrocompetent cells using an *E. coli* Pulsar electroporation unit and the protocol provided by the manufacturer (Bio-Rad). The cells were plated onto LB-Km agar and incubated overnight at 37 °C. Km-resistant colonies were then streaked for isolation onto LB-Km agar, and DNA was isolated from LB-Km overnight cultures using the Promega Genomic DNA Purification Kit. The presence of the desired mutation was confirmed by PCR analysis using primers PR48 and PR49 (Table 1) specific for the *lamB* sequence outside of the deletion site.

Construction of plasmid pKS34 carrying *lamB* under the control of an arabinose-inducible promoter, *ParaBAD* : : *lamB*—Chromosomal DNA isolated from *S. Typhimurium* SL1344 (Wizard Genomic DNA Purification Kit; Promega) served as template for high-fidelity PCR amplification (Platinum PCR SuperMix High Fidelity; Invitrogen) of the *lamB* gene using the forward primer PR48 and the reverse primer PR49 (Table 1). The 1.4 kb PCR product was purified and ligated into pGEM-T Easy (Promega), and *E. coli* JM109 competent cells (Promega) were transformed with this, following the manufacturer's protocols. Plasmid minipreps (Wizard *Plus* Minipreps; Promega) from the ampicillin-resistant transformants were screened by PCR using primers PR48 and PR29 (Table 1) to determine that the insert was oriented in the same direction as the *lacZ* gene in order to facilitate cloning into pBAD33. The subsequent plasmid was designated pKS33.

Digestion of pKS33 with *SacI* and *SphI* yielded a 1.5 kb fragment carrying the *lamB* gene. This fragment was isolated using agarose gel electrophoresis, extracted (Freeze 'N Squeeze columns; Bio-Rad) and cleaned (Wizard DNA Clean-Up System; Promega). It was then ligated with *SacI/SphI*-digested pBAD33 (a generous gift from Dr Jon Beckwith, Harvard University, Boston, MA, USA), and *E. coli* JM109 cells (Promega) were transformed with it and plated onto LB-Cm plates. Plasmid minipreps (Wizard *Plus* Minipreps; Promega) from transformant colonies were screened for the *lamB* insert by digestion with *SacI* and *SphI*, and agarose gel electrophoresis. The resultant plasmid was designated pKS34.

Overexpression of LamB and σ^E activation—To measure the effect of LamB overproduction on σ^E activation as measured from a σ^E -dependent promoter in pTF-p2 we transformed SMS760 (SL1344/pTF-p2) with pKS34 to produce strain SMS908 (Table 1). The desired strains were grown overnight in LB plus the appropriate antibiotic. Overnight cultures were then diluted 1 : 100 into fresh LB broth with the appropriate antibiotic(s), and grown to mid-exponential phase before being divided into six separate cultures to which 0, 25, 50, 100, 200 or 400 μ M L-arabinose was added. Cultures were then incubated for an additional 1 h at 37 °C with aeration, at which point β -galactosidase activity was assayed using the method of Miller (1992).

RESULTS AND DISCUSSION

Shifts from glucose to maltose, citrate or succinate result in a sustained increase in σ^E activity

In *S. Typhimurium*, a number of C-source utilization enzymes and systems are induced during the transition from growth to C-starvation in an effort to scavenge any and all available C-sources, and avoid entry into the physiologically more drastic 'survival' mode (Spector, 1998). Many of the utilization systems involve the induction of OM, PP and/or IM components in addition to cytoplasmic proteins. An example of an OM component of an alternative C-source utilization system is LamB, which functions as a porin-like protein involved in the uptake of maltose and maltodextrins (Schulein & Benz, 1990; Hall *et al.*, 1997). Induction of a *lamB* : *lacZ* fusion during C-starvation, as well as during shifts from glucose to maltose, showed that *lamB* is induced approximately 17-fold by C-starvation (1.4 $\pm$ 0.4 Miller units in exponential-phase cells compared to 23.9 $\pm$ 2.4 Miller units in 5 h C-starved cells), and approximately 46-fold during shifts from glucose to maltose (1.5 $\pm$ 0.7 Miller units in cells prior to the shift to maltose compared to 70.1 $\pm$ 1.7 Miller units 4 h after the shift to maltose). These results, along with previous findings that σ^E is a key regulator of the SSR and is activated within the first few hours of C-starvation (Kenyon *et al.*, 2002), led us to examine the role of σ^E early in the SSR during the 'avoidance' phase. For this, we assessed σ^E activity during the transition from growth on glucose to less-preferred but utilizable C-sources in *S. Typhimurium*.

Fig. 1(a) illustrates the typical increase in σ^E activity (as determined by increased β -galactosidase expression driven by an σ^E -dependent promoter) observed during C-starvation (Kenyon *et al.*, 2002), and is included for comparison purposes. Shifts from glucose to maltose, citrate or succinate all produced discernible, yet transient, diauxic lag periods, and

more importantly sustained increases in σ^E activity (Fig. 1b–d) comparable to C-starved cells (Fig. 1a) over the same time period. In contrast, shifts from glucose to maltose, citrate, succinate or any other alternative C-source tested, as well as C-starvation, produced relatively constant β -galactosidase activity [2478 ± 69 (SEM) Miller units] over the same time period from the σ^E -independent (σ^{70} -dependent) promoter in pTF-p1.

A common feature of the utilization systems for these C-sources appears to be the presence of an OM- and/or PP-protein component in addition to IM transporters. In *S. Typhimurium*, this includes the LamB OM-maltoporin complex and the MalE extracytoplasmic solute receptor (binding protein) for maltose utilization (Schulein & Benz, 1990; Hall *et al.*, 1997; Lawhon *et al.*, 2003). Utilization of citrate (a C₆-tricarboxylate) involves an extracytoplasmic (PP) solute-receptor (binding) protein without a specific OM component. TctC is the tricarboxylate-binding-receptor component of the TctABC tripartite-tricarboxylate transporter (TTT) of *S. Typhimurium* involved in citrate utilization (Winnen *et al.*, 2003).

The scenario for succinate utilization in *S. Typhimurium* is more complicated. The homologues of the *dctPQM* genes encoding the aerobic dicarboxylate-transporter system DctPQM in *Rhodobacter capsulatus* (Rabus *et al.*, 1999; Janausch *et al.*, 2002) are apparently absent from the *S. Typhimurium* genome (McClelland *et al.*, 2001). However, there are at least three hypothetical genes encoding putative extracytoplasmic solute receptors for C₄-dicarboxylates that may play a role in succinate utilization (STM3169, GenBank accession number AE008845,17756–18746; *yiaO*, accession number AE008870, 9637–10623; STM4054, accession number AE008889, complement 10233–11216). Of these three, YiaO along with YiaM/YiaN, and STM4054 along with STM4052/STM4053 may constitute tripartite ATP-independent periplasmic- (TRAP) transporter systems involved in succinate utilization (Rabus *et al.*, 1999; Janausch *et al.*, 2002). In addition, *S. Typhimurium* possesses the genes for two other C₄-dicarboxylate-transport systems: (1) the anaerobic Dcu dicarboxylate-transporter system, which appears to be involved in fumarate respiration rather than C₄-dicarboxylate utilization and as part of this function actually results in succinate efflux from the cytoplasm (Janausch *et al.*, 2002), and (2) a homologue of the DctA H⁺/Na⁺: C₄-dicarboxylate/dicarboxylic amino acid symporter, which was found to be a major C₄-dicarboxylate transporter during aerobic growth in *E. coli*. Mutants of *dctA* in *E. coli* grow very poorly or not at all on succinate and other C₄-dicarboxylates (Davies *et al.*, 1999). However, its cognate extracytoplasmic solute receptor (i.e. succinate-binding protein) has not been definitively identified. In *E. coli*, the *cbt* gene was proposed as the putative C₄-dicarboxylate-binding protein possibly as part of an OM porin (Lo, 1977; Lo & Bewick, 1981). Whether this complex is expressed under the conditions tested in this study has not been demonstrated, but recent evidence suggests that *cbt* may be an allele of the ferric-enterobactin-transport component gene *fepA*, which is iron regulated (Braun & Braun, 2002) in both *E. coli* and *Salmonella*. Thus, the involvement of PP and/or OM component(s) in succinate utilization in *S. Typhimurium*, although likely, is ambiguous and clearly complicated.

Shifts from glucose to the PEP : PTS-dependent sugars trehalose, mannose, mannitol or fructose do not result in a sustained increase in σ^E activity

Many sugars are translocated into the cell by the phosphoenolpyruvate (PEP) : sugar phosphotransferase system (PTS) (Postma *et al.*, 1993). The utilization of secondary PTS sugars, and some other non-PTS sugars, is catabolite repressed and/or subject to inducer exclusion during growth on glucose. This is in part a result of low intracellular levels of the signal nucleotide cyclic AMP (cAMP) caused by greatly reduced adenylate cyclase activity, resulting from greatly reduced levels of the phosphorylated Enzyme IIA component of the glucose-specific PTS (P~EnzIIA^{gluc}). The exhaustion of glucose leads to accumulation of P~Enz IIA^{gluc} and the activation of adenylate cyclase. The resulting increased cAMP levels, and in some cases the presence of the C-source (or a derivative), induces the expression of the utilization enzymes for the secondary C-source. For some of these C-sources, this involves the production of specific Enzyme IIA and IIB components for its PTS. The IIB component for these PTS sugars is an IM protein, while their IIA component is associated with the cytoplasmic side of the IM; however, there are no PP or OM components involved in the PTS. We therefore wanted to determine if shifts from glucose to known PTS sugars might also generate signals leading to sustained σ^E activation.

In contrast to maltose, citrate and succinate, a shift from glucose to the PTS-dependent sugars trehalose (Fig. 2a), fructose (Fig. 2b), mannose or mannitol (data not shown) did not lead to a discernible diauxic lag period or sustained σ^E activation. Results for mannose and mannitol were very similar to that of fructose. These results indicate that a shift from glucose to a secondary C-source, in general, is insufficient to cause sustained σ^E activation. It also demonstrated more specifically that shifts from glucose to PTS sugars do not generate signals leading to sustained σ^E activation, thus supporting our hypothesis, given that their utilization does not involve PP and/or OM components.

Shifts from glucose to the non-PTS C-sources melibiose, l-arabinose, d-galactose or glycerol do not result in a sustained increase in σ^E activity

Some secondary C-sources do not require the PTS for their utilization, but instead rely on a specific IM-associated transporter, permease or channel to bring them into the cytoplasm for utilization; although they may exhibit catabolite repression and/or inducer exclusion (as described above). Moreover, in some cases, their expression in *S. Typhimurium* is uncertain. Examples of non-PTS C-sources that exhibit these characteristics include glycerol, L-arabinose, D-galactose and melibiose in *S. Typhimurium* (Postma, 1977; Muller *et al.*, 1985; Benner-Luger & Boos, 1988; Horazdovsky & Hogg, 1989; Kehres & Hogg, 1992; Moat *et al.*, 2002). Thus, we examined whether shifts from glucose to one of these C-sources would result in a sustained increase in σ^E activity.

Neither shifts from glucose to melibiose (Fig. 3a), L-arabinose (Fig. 3b), D-galactose, nor glycerol (data not shown) resulted in a sustained increase in σ^E activity; although there was a transient increase in some cases. The results for D-galactose and glycerol were very similar to the result for L-arabinose. Interestingly, the shift from glucose to melibiose produced an observable transitory diauxic lag period, but again this was not accompanied by a sustained

elevation in σ^E activity (Fig. 3a). These latter findings suggest that it is not the occurrence of a diauxic lag period that results in signals leading to a sustained rise in σ^E activity.

In *E. coli*, there appears to be a high-affinity arabinose-transport system encoded by the *araFGH* operon, with AraF being an arabinose-binding protein and AraGH composing an IM-transport system (Horazdovsky & Hogg, 1989; Kehres & Hogg, 1992). However, the *araFG* genes are not present in the *S. Typhimurium* genome (McClelland *et al.*, 2001; GenBank accession number AE006468 or NC_003197). This supports our hypothesis since the PP-binding protein for arabinose AraF would not be produced. Database analysis of the *S. Typhimurium* LT-2 genome sequence did not indicate known or putative AraF homologues.

In both *E. coli* and *S. Typhimurium*, D-galactose can be transported into the cell through the galactose-specific galactose permease GalP or the binding-protein-dependent methyl-galactoside Mgl system (Postma, 1977; Muller *et al.*, 1985; Benner-Luger & Boos, 1988). The *mglB*-encoded protein is a PP-binding protein that can bind to galactose as well as other sugars, e.g. fucose. Although this might appear contradictory to our hypothesis, galactose only slightly induces the *mgl* genes in a *galP*⁺ strain background (Postma, 1977; Muller *et al.*, 1985; Benner-Luger & Boos, 1988). Thus, the MglB protein is not induced significantly under our experimental conditions. So, the lack of a sustained increase in σ^E activity during a glucose to D-galactose shift can still be explained in the context of our hypothesis. The pertinent characteristics for all the secondary C-sources tested are summarized in Table 2.

Inability to produce the LamB protein reduces the activation of σ^E during a shift from glucose to maltose

As stated previously, a common feature of maltose, citrate and succinate utilization appears to be the production of an OM and/or extracytoplasmic (PP) solute-receptor component(s) involved in their utilization, e.g. the LamB OM-porin-like protein complex (Schulein & Benz, 1990), or the extracytoplasmic solute receptors involved in C₄-dicarboxylate and C₆-tricarboxylate transport (Widenhorn *et al.*, 1988; Rabus *et al.*, 1999; Janausch *et al.*, 2002; Winnen *et al.*, 2003). Another possible factor involved in σ^E activation under these conditions might be a slower growth rate produced by the secondary C-source, e.g. shifts to citrate or succinate (Fig. 1b–c). To investigate this, we created a targeted deletion of the *lamB* gene to knock-out LamB production.

We targeted the *lamB* gene for several reasons. First, it is not essential for the utilization of maltose, yet it is induced during shifts from glucose to maltose (discussed above). Second, a *lamB* : : Ω -Km mutant strain (SMS871) exhibits virtually identical growth, compared to the wild-type parent strain, during glucose to maltose shifts (Fig. 4a). Furthermore, the situation for citrate or succinate utilization is problematic for this study. Mutants of *tctC*, lacking the extracytoplasmic tricarboxylate-binding-protein/receptor component, are defective in citrate utilization (Winnen *et al.*, 2003), and thus may mimic conditions of C-starvation. What's more, succinate utilization in *Salmonella* is not very well characterized. There are at least three putative C₄-dicarboxylate-binding proteins in the *S. Typhimurium* genome (McClelland *et al.*, 2001), and the presence of C₄-dicarboxylate PP- and/or OM-utilization components in *S. Typhimurium* as well as *E. coli* still requires clarification. Deletion of one

or more of the C₄-dicarboxylate PP-binding proteins is likely to make the cells defective in succinate utilization similar to the scenario for citrate utilization.

As would be predicted from our hypothesis, the fold-induction of the σ^E -dependent promoter is reduced in a *lamB* knock-out mutant to ~50 % of the fold-induction levels seen in the wild-type parent: a twofold-induction compared to a fourfold-induction, respectively (Fig. 4b). Residual activation of σ^E may be the result of the production of MalE, the extracytoplasmic maltose-binding protein/receptor required for maltose utilization, as well as other maltodextrin-utilization proteins that are also expressed under these conditions (Hall *et al.*, 1997). A previous study in *E. coli* found that at lower levels of expression a folding-defective MalE protein is degraded by DegP or Protease III (Betton *et al.*, 1998), supporting this idea. Unfortunately, knocking-out a required utilization component, such as MalE, would prevent growth on the C-source, and would again mimic C-starvation. But the fact that the loss of LamB production stifles the increase in σ^E activity normally seen during glucose to maltose shifts, yet the *lamB* mutant exhibits virtually indistinguishable growth rates under these conditions (Fig. 4a), supports the hypothesis that one or more σ^E -regulated functions are needed for efficient LamB incorporation into the OM during this adaptation process.

Overproduction of LamB protein in the absence of glucose starvation or a glucose to maltose shift increases activation of σ^E

To further demonstrate that up-regulated expression of LamB directly results in increased σ^E activity, we overproduced LamB from the arabinose-inducible *araBAD* promoter in pBAD33 (Guzman *et al.*, 1995). As shown in Fig. 5, increased σ^E activity was observed when we artificially overproduced LamB from an arabinose-inducible promoter (SMS908) in the absence of glucose starvation or during a glucose to maltose shift (Fig. 5b). An increase in σ^E activity was not observed under these conditions in a strain lacking the pKS34 plasmid (SMS760), indicating that it is the overproduction of LamB and not the addition of arabinose itself that results in increased σ^E activity. Furthermore, the increased levels of β -galactosidase activity observed with rising concentrations of arabinose in SMS908 (Fig. 5b) required a functional σ^E protein, since in similar experiments with the *rpoE*: Ω -Km^r mutant SMS909 low but constant β -galactosidase activities (approximately 10 Miller units), equivalent to a strain carrying the pRS1274 vector alone, were detected.

These results demonstrate that the inappropriate overproduction of LamB can also generate signals that result in a sustained increase in σ^E activity. This is not that surprising since the artificial or inappropriate overproduction of several other outer-membrane proteins (OMPs), including OmpC or OMP-like C-terminal peptides, can up-regulate σ^E activity through increased DegS-mediated proteolysis (Ades *et al.*, 1999; Walsh *et al.*, 2003). An interesting difference in LamB is the absence of the Y-X-F [Tyr-X-Phe; where X can be several different amino acids such as Tyr (Y) or Gln (Q)] C-terminus that OmpC and several other OMPs possess. This is proposed to be the peptide signal/ligand that binds to the PDZ-domain of DegS, activating its protease activity (Walsh *et al.*, 2003). Interestingly, the LamB protein (accession no. NP_463096) possesses a Q-M-E-I-W-W (Gln-Met-Glu-Ile-Trp-Trp) C-terminal pentapeptide that exhibits some similarity to the Φ -X- Φ [where Φ is a

hydrophobic residue such as Val (V), Leu (L), Ile (I), Met (M) or Pro (P)] ligand for the proposed class II PDZ-binding site (Ponting, 1997; Songyang *et al.*, 1997); although the closest match is the M-E-I sequence two residues distal to the C-terminus of the protein. This suggests that LamB generated-signals may differ, or work through different pathways from the typical C-terminal peptide signal or DegS-dependent pathways reported to result in σ^E activation. Intriguingly, the C-terminal pentapeptide of the tricarboxylate-binding receptor TctC (F-G-L-A-K, Phe-Glu-Leu-Ala-Lys; accession no. AAG31205) and the putative dicarboxylate-binding receptors STM3169 (Y-I-E-N-M, Tyr-Ile-Glu-Asn-Met; accession no. NP_462084), YiaO (V-D-E-V-Q, Val-Asp-Glu-Val-Gln; accession no. NP_462573) and STM4054 (Q-T-A-K-N, Gln-Thr-Ala-Lys-Asn; accession no. NP_462935) are also lacking the typical OMP-like C-terminal peptide sequence, again suggesting that alternative signals or DegS-independent pathways may be involved.

A question arises as to what σ^E -regulated functions might be involved in this adaptation process. We are currently searching for these functions. We do not believe them to be either DegP, FkpA or SurA since we have found that in *S. Typhimurium* they are not regulated by σ^E (or C-starvation) under the conditions tested here (W. J. Kenyon, A. Stevenson, S. Humphreys, M. Roberts & M. P. Spector, unpublished data). Thus, some alternative protease(s), chaperone(s), peptidyl-prolyl isomerase(s) or other function(s) as-yet-unidentified, would appear to be involved in the adaptation of the cell envelope to C-starvation conditions.

Studies in *E. coli* (Ades *et al.*, 2003) identified trends in σ^E activity occurring before, during and after the inducing stress of high temperature. During the initiation of the stress response, σ^E activity is elevated. The activity remains elevated during the adaptation phase while the cell adapts to the stress. When the stress is removed, σ^E activity drops below that seen in normal conditions, and then slowly rises to pre-stress levels. A similar scenario can be postulated for shifts from glucose to maltose, for example, which even though not typically considered a stress do require the cells to adapt to the change in growth conditions. Interestingly, during C-starvation σ^E levels are seen to continue to rise over a 48–72 h period before levelling off (Kenyon *et al.*, 2002).

Conclusions

We report here that shifts from glucose to maltose, citrate or succinate, but not trehalose, mannose, mannitol, fructose, glycerol, L-arabinose, galactose or melibiose, result in a sustained elevation of σ^E activity. The common feature of maltose, citrate and succinate utilization is the production of extracytoplasmic (PP) solute-receptors/binding proteins (e.g. TctC) and/or OM transport proteins or porins (e.g. LamB). The other secondary C-sources tested required either PEP : PTS-dependent-transport systems (e.g. trehalose, mannose, mannitol and fructose) or non-PTS IM-transport systems (e.g. glycerol, L-arabinose, galactose and melibiose). However, an extracytoplasmic component in their utilization systems is either absent, unknown or not expressed under the conditions tested in *S. Typhimurium*. The findings presented here support the hypothesis that σ^E -regulated functions are needed for the adaptation of cells to new environmental conditions that necessitate changes in the extracytoplasmic compartment of the cell, i.e. OM or periplasm.

This implies that members of the σ^E regulon have functions beyond the repair or elimination of damaged extracytoplasmic proteins resulting from an environmental stress.

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Fig. 1.

Growth and σ^E activation during shifts from glucose to (a) no secondary C-source (early C-starvation), (b) maltose, (c) citrate and (d) succinate. Graphs represent growth (OD_{600}) (●) and σ^E activation (■) as reported by measuring *lacZ* expression (β -galactosidase activity) driven by a σ^E -dependent promoter. β -Galactosidase activity is monitored using the method of Miller (1992) and is given in Miller units. Data for β -galactosidase activities represent means $\pm$ SEM (n 3).

Fig. 2.

Growth and σ^E activation during shifts from glucose to (a) trehalose and (b) fructose. Graphs represent growth (OD_{600}) (•) and σ^E activation (■) as reported by measuring *lacZ* expression (β -galactosidase activity) driven by an σ^E -dependent promoter. Promoter activity is reported by measuring *lacZ* expression (β -galactosidase activity) driven by the σ^E -dependent *rpoE* promoter. β -Galactosidase activity is monitored using the method of Miller (1992) and is given in Miller units. OD_{600} values above 2.0 are not plotted. Data for β -galactosidase activities represent means $\pm$ SEM (n = 3).

Fig. 3.

Growth and σ^E activation during shifts from glucose to (a) melibiose and (b) L-arabinose. Graphs represent growth (OD_{600}) (•) and σ^E activation (■) as reported by measuring *lacZ* expression (β -galactosidase activity) driven by a σ^E -dependent promoter. β -Galactosidase activity is monitored using the method of Miller (1992) and is given in Miller units. OD_{600} values above 2.0 are not plotted. Data for β -galactosidase activities represent means $\pm$ SEM (n = 3).

Fig. 4.

Effect of a *lamB* knock-out mutation on σ^E activation during shifts from glucose to maltose utilization. (a) Growth (inset) and (b) σ^E activation, reported as fold-induction from a σ^E -dependent promoter, during shifts from glucose to maltose in the wild-type *lamB*⁺ strain (SMS760) (•) and *lamB* mutant derivative (SMS874) (○). Time zero represents the initiation of the diauxic lag period. Fold-induction was calculated by dividing the value at each time point by the value at time zero. Promoter activity is reported by measuring *lacZ* expression (β -galactosidase activity) driven by the σ^E -dependent *rpoE* promoter. β -Galactosidase activity was determined using the method of Miller (1992). Data presented represent mean fold-inductions from at least three independent experiments. The SEM for the data points is 24 % of the mean.

Fig. 5.

Effect of overproduction of LamB in LB medium. (a) Photograph of strains *rpoE*⁺(pKS34, pRS1274) (1), *rpoE*⁺(pKS34, pTF-p2) (2) and *rpoE*⁻: Ω-Km^r(pKS34, pTF-p2) (3). Strains were streaked onto MacConkey-lactose agar, and a disk saturated with 0.25 % (w/v) L-arabinose was placed in the centre of the plate. Production of β-galactosidase activity is indicated by the appearance of a red colour (seen here as dark grey) in the streak and the agar surrounding the streak. (b) β-Galactosidase activity from pTF-p2 alone (SMS760, •), and from pTF-p2 in response to increasing amounts of arabinose-induced expression of LamB from pKS34 (SMS908, ○). β-Galactosidase activity is monitored using the method of Miller (1992) and is given in Miller units. Data for β-galactosidase activities represent means±SEM (n 3).

Table 1

Bacterial strains, plasmids and primers

Strain, plasmid or primer	Relevant characteristic *	Source/reference
Strains		
SL1344	<i>hisG46</i> (mouse- virulent <i>S. Typhimurium</i>)	Hoseith & Stocker (1981)
ST55	SL1344 <i>lamB</i> :: MudI (<i>lac</i> Km ^r)	This study
ST213	SL1344 <i>rpoE</i> :: Ω- Km ^r	Kenyon <i>et al.</i> (2002)
ST276	Wild-type <i>S. Typhimurium</i> NCTC12023/pKD46 (Ap ^r)	Mark Roberts [‡]
SMS758	SL1344/pRS1274 (Ap ^r)	Kenyon <i>et al.</i> (2002)
SMS759	SL1344/pTF-p1 (Ap ^r)	Kenyon <i>et al.</i> (2002)
SMS760	SL1344/pTF-p2 (Ap ^r)	Kenyon <i>et al.</i> (2002)
SMS865	ST276 <i>lamB</i> :: Ω- Km ^r	This study
SMS871	SL1344 <i>lamB</i> :: Ω- Km ^r	This study
SMS872	SMS871/pRS1274 (Ap ^r)	This study
SMS873	SMS871/pTF-p1 (Ap ^r)	This study
SMS874	SMS871/pTF-p2 (Ap ^r)	This study
SMS907	SMS758/pKS34 (Cm ^r)	This study
SMS908	SMS760/pKS34 (Cm ^r)	This study
SMS909	ST213/pTF-p2-pKS34 (Ap ^r Cm ^r)	This study
Plasmids		
pRS1274	Vector for making transcriptional <i>lacZ</i> fusions (Ap ^r)	Simons <i>et al.</i> (1987)
pTF-p1	<i>rpoEp1</i> :: <i>lacZ</i> in pRS1274 (<i>rpoE</i> -independent promoter; Ap ^r)	Testerman <i>et al.</i> (2002)
pTF-p2	<i>rpoEp2</i> :: <i>lacZ</i> in pRS1274 (<i>rpoE</i> -dependent promoter; Ap ^r)	Testerman <i>et al.</i> (2002)
pKD46	Carries λ-Red γ, β and Exo genes under the control of an arabinose-inducible <i>araBAD</i> promoter, P _{<i>araBAD</i>} (Ap ^r)	Datsenko & Wanner (2000)
pKD4	Carries FRT-Km ^r -FRT Ω-cassette (Ap ^r)	Datsenko & Wanner (2000)
pBAD33	Vector for cloning genes under the control of P _{<i>araBAD</i>} (Cm ^r)	Guzman <i>et al.</i> (1995)
pKS33	1.4 kb fragment carrying <i>lamB</i> in pGEM-TEasy ® (Ap ^r)	This study
pKS34	<i>SacI</i> – <i>SphI</i> fragment from pKS33 with <i>lamB</i> ⁺ in pBAD33 (Cm ^r)	This study
Primers		
PR18	CCATCAGATCCCGAATAATCCAATGTCTCTCCC Sequence (5' to 3'):	

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Strain, plasmid or primer	Relevant characteristic*	Source/reference
PR29	GTTTCCCAAGTCACGAC	
PR48	AGGTGGCAGCGTTTAAAGAA	
PR49	GCCCTCGTTACGTCAGAT	
PR122	AGGTGGCAGCGTTTAAAGAAAAGCAATGATCTCAGGAGATGTGTAGGGCTGGAGCTGCTTC	
PR123	GCCCCTCGTTACGTCAGATGACCATCGTATTACCACACAGACATATGAATATCCTCCTTA	

* Km^r, Kanamycin resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

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Table 2

Summary of the pertinent characteristics of the secondary C-sources

C-source shifted to	PTS sugar [*]	PP-BP [†]	OMP [‡]	Diauxic lag	Sustained σ^E activation
Maltose	–	+	+	+	+
Citrate	–	+	–	+	+
Succinate	–	+	–	+	+
Trehalose	+	–	–	–	–
Mannose	+	–	–	–	–
Mannitol	+	–	–	–	–
Fructose	+	–	–	–	–
Melibiose	–	–	–	+	–
Glycerol	–	–	–	–	–
L-Arabinose	–	NK	–	–	–
Galactose	–	NK	–	–	–

^{*} PTS sugar, utilization of C-source requires the PEP-dependent : PTS system.

[†] PP-BP, utilization system has a PP-binding protein component; NK, not known to be present in *S. Typhimurium* genome (accession no. AE006468 or NC_003197, *Salmonella typhimurium* LT-2 complete genome sequence) or not known to be expressed under the conditions being tested.

[‡] OMP, utilization system has an OMP component.