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The LysR Homolog LrhA Promotes RpoS Degradation by Modulating Activity of the Response Regulator SprE

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Synthesis of the OmpF porin of *Escherichia coli* is regulated in response to environmental and growth phase signals. In order to identify constituents of the various regulatory pathways involved in modulating *ompF* transcriptional expression, transposon insertion mutagenesis was performed and mutations that increased *ompF′-lacZ* activity were identified as previously described. Mutations mapping to a previously identified gene of unknown function, *lrhA*, were obtained. We found that LrhA, a LysR homolog, functions as a regulatory component in the RpoS-dependent growth phase repression of *ompF*. In addition to altered growth phase regulation of *ompF*, these *lrhA* mutants have pleiotropic stationary-phase defects as a result of decreased RpoS levels. We provide evidence that LrhA promotes degradation of RpoS by functioning within a genetic pathway that includes the response regulator SprE and the ClpXP protease. LrhA functions upstream of the other components in the pathway and appears to modulate the activity of SprE.

Physiological adaptation to changing environmental conditions is essential to microbial viability. This is particularly important for bacteria that alternatively inhabit distinct niches differing in conditions of oxidative stress, temperature, pH, and osmolarity, as well as nutrient availability and exposure to various toxins. OmpF and OmpC, the major porins of the gram-negative bacterium *Escherichia coli*, allow nonspecific diffusion of solutes through the protective outer membrane. Because the abundant OmpF porin allows for the more rapid diffusion of solutes through the outer membrane, it is a major entry point for most molecules that *E. coli* encounters, contributing significantly to outer membrane permeability and thus susceptibility to environmental hazards (33). For this reason, regulation of OmpF synthesis is necessarily complex and responsive to nutrient limitation (27) and a variety of environmental conditions (34), the best understood being medium osmolarity (10, 11, 28, 35). Since OmpF synthesis is highly responsive to fluctuation in various environmental parameters, studying its regulation provides us with an opportunity to understand the sensing mechanisms and complex regulatory networks necessary for *E. coli* adaptive physiology.

Synthesis of OmpF is responsive to the bacterial growth rate such that during rapid logarithmic growth OmpF levels are high, but as bacterial growth begins to be limited by nutrient availability, OmpF synthesis decreases (27). This, and many other stationary-phase-dependent alterations in envelope physiology, presumably aids in survival under conditions of decreased metabolic capacity by encapsulating the bacterium within a protective, less permeable cell envelope (19). In addition to protective envelope adaptations, synthesis of a core set of cytoplasmic proteins is induced, independently of the nature of the limiting nutrient, as *E. coli* enters into stationary phase, resulting in a multiple-stress-resistant physiological state. For instance, stationary-phase *E. coli* is resistant to heat and osmotic and oxidative shock, as well as treatment with alkylating agents, ethanol, and acidic or basic pH (24, 27). These protective functions (22, 27), and in particular the stationary-phase-dependent decrease in OmpF synthesis (27, 36), were found to be mediated by the alternative primary sigma factor σ^S (σ^{38}), encoded by the *rpoS* gene. RpoS-dependent stationary-phase regulation of OmpF synthesis is mediated at the transcriptional level, as indicated by the approximately three- to fivefold derepression of *ompF′-lacZ* expression observed in an *rpoS* null mutant specifically during stationary-phase growth (36). However, the precise mechanism by which RpoS promotes transcriptional repression at *ompF* is unclear.

RpoS functions as a global regulator of many genes required for the physiological transition into stationary-phase growth, and as such, its levels are highly responsive to the bacterial growth rate. During rapid growth, RpoS is maintained at a low level. However, during the transition to stationary phase, RpoS levels rise dramatically. This strict growth phase regulation of RpoS accumulation is quite complex in that it has been proposed to be mediated at all levels of synthesis and stability and via multiple inputs (15). Expression of various *rpoS′-lacZ* operon fusions increases approximately four- to fivefold during entry into stationary phase in rich medium and is weakly induced in response to starvation for carbon, nitrogen, or phosphate in minimal medium (22). The weak starvation induction ratio seen with *rpoS′-lacZ* transcription fusions is in stark contrast to the strongly increased amount of RpoS observed under the same medium conditions (20), implying that there is significant posttranscriptional growth phase regulation of RpoS. In fact, a greater stationary-phase induction ratio of RpoS at the level of translation was demonstrated with various *rpoS′-′lacZ* protein fusions (20, 23, 26).

However, it has recently been argued that the stationary-phase-dependent induction of RpoS occurs exclusively through regulation of its susceptibility to proteolysis (46). Logarithmic-phase RpoS accumulation is minimal due to rapid degradation by the ClpXP protease, which requires amino acid residues 173 to 188 of RpoS for target recognition (40). ClpXP-mediated degradation of RpoS absolutely requires the two-component response regulator SprE (36), also called RssB (29) or, in *Salmonella typhimurium*, MviA (1). Constitutively active alleles of *sprE* prevent full induction of RpoS as bacteria enter stationary phase (36), while *sprE* null mutants allow logarithmic-phase RpoS accumulation to levels nearly equivalent to those observed in stationary phase (29, 36). SprE functions upstream

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TABLE 1. Bacterial strains

Strain	Genotype	Reference[a]
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 ptsF25 deoC1 thiA1*	7
KEG400	MC4100 *ompR107* Φ(*ompF′-lacZ*$^+$)16-13	
KEG403	KEG400 *lrhA49*::*cam*	
KEG404	KEG400 *rpoS*::*kan*	
KEG405	KEG403 *rpoS*::*kan*	
KEG408	RO91 *lrhA49*::*cam*	
KEG409	RO91 *lrhA*::*spc*	
KEG410	KEG408 *lrhA49*::*cam*::*spc*	
KEG415	LP801 *lrhA49*::*cam*	
KEG416	LP801 *lrhA*::*spc*	
KEG418	MC4100 *lrhA49*::*cam*	
KEG419	MC4100 *lrhA*::*spc*	
KEG420	LP810 *lrhA*::*spc*	
KEG421	KEG408 *clpX*::*kan*	
KEG422	KEG409 *clpX*::*kan*	
KEG423	KEG412 *rpoS*::*kan*	
KEG429	MC4100 *clpX*::*kan clpP*::*cam*	
KEG445	ZK916 *lrhA49*::*cam*	
KEG446	ZK916 *lrhA*::*spc*	
KEG448	KEG445 *lrhA49*::*cam*::*spc*	
KEG449	LP867 *lrhA49*::*cam*	
KEG447	LP867 *lrhA*::*spc*	
LP801	RO91 *sprE*::IS*1 trpC*::Tn*10*	36
LP810	RO91 *sprE19*::*cam*	36
LP855	RO91 *clpX*::*kan clpP*::*cam*	36
LP867	ZK916 *cysC*::Tn*10 rpoS*::*kan*	37
RO91	MC4100 λΦ(*rpoS742′-′lacZ*)	20
ZK916	MC4100 Φ(*bolA′-lacZ*)	21

[a] Strains for which no reference is given were created in this study.

of ClpXP to promote RpoS degradation in a substrate-specific manner which leaves ClpXP protease activity unaltered (36). Thus, SprE is the first response regulator to be implicated in regulating protein degradation, and its novel C-terminal domain reflects this unique function.

For all known two-component response regulators, activity is modulated through phosphorylation at a conserved aspartic acid residue (16, 43). The N-terminal receiver domain of SprE contains this conserved residue, and so SprE activity is also predicted to be modulated via phosphorylation. The phosphorylation of SprE at the conserved aspartic acid has been demonstrated in vitro with acetyl phosphate (5). Additionally, it has been shown that Δ(*pta ackA*) mutants, which can no longer synthesize acetyl phosphate, affect the in vivo function of SprE such that it no longer efficiently promotes RpoS degradation (5). This implies that it is the phosphorylated form of SprE that is functionally active. In the absence of acetyl phosphate, however, RpoS accumulation remains growth phase regulated, indicating that there must be an additional factor(s) that influences SprE activity. The additional growth phase signal(s) and effector molecule(s) that regulate SprE remain to be discovered. Here, we describe the identification and initial characterization of another component of the SprE/ClpXP pathway that functions to modulate SprE activity.

MATERIALS AND METHODS

Bacteria and bacteriophage. All bacterial strains constructed for this study (Table 1) are derivatives of MC4100 [F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*] (7). Bacteria were grown at 37°C, and standard microbiological techniques were used for strain construction (41). Genetic map positions were determined by standard techniques, including Hfr mapping and P1*vir* transduction.

Media, reagents, and enzymes. Luria-Bertani (LB) growth medium was prepared as described previously (41). ONPG (*ortho*-nitrophenyl-β-D-galactoside) utilized for β-galactosidase assays was purchased from Sigma. β-Agarase (New England Biolabs), T4 DNA ligase (New England Biolabs), *Taq* polymerase (United States Biochemical Corp.), and reagents used for DNA sequence analysis (United States Biochemical Corp.) were used according to the recommendations of the respective manufacturers. For immunoblot analysis, proteins were transferred to a nitrocellulose membrane (pore size of 0.2 μm) purchased from Schleicher & Schuell. Monoclonal antibodies to RpoS were a gift of R. Burgess (32). Rabbit antibodies to ClpX and ClpP were a gift of S. Gottesman (12, 25). The ECL antibody detection kit and the anti-mouse and anti-rabbit immunoglobulin horseradish peroxidase-linked whole antibody (from sheep) were purchased from Amersham. Synthesis of oligonucleotide primers was provided by the Princeton University Department of Molecular Biology synthesis facility.

Biochemical analysis. β-Galactosidase assays were performed as previously described (42). Activities are expressed as (units/A_{600}) × 10^3, where 1 unit is 1 μmol of orthonitrophenol formed per minute. The data are averages of at least four independent assays, and the standard deviations are indicated as error bars.

For immunoblot analysis, whole-cell extracts were prepared by pelleting the bacterial culture and resuspending the pellet in a volume of loading buffer (41) that was determined to standardize for total protein based on culture optical density at 600 nm (OD_{600}). The samples were boiled for 10 min, and an equal volume of sample was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Immunoblot analysis was performed as described previously (32). The anti-RpoS monoclonal antibody was used at a dilution of 1:1,000, and the anti-ClpP and anti-ClpX rabbit polyclonal antibodies were used at a dilution of 1:5,000.

PCR amplification, DNA sequence analysis, and DNA cloning. Chromosomal DNA adjacent to the *lrhA49*::*cam* insertion was amplified by a two-round PCR procedure (6). The first round of PCR (30 cycles) was performed at low stringency (annealing temperature of 45°C) with a primer recognizing the *cat* gene within mini-Tn*cam* (out1-L, 5′CAGGCTCTCCCCGTGGAG) and a set of degenerate primers with a 5′ tag (ARB1, 5′GGCCACGCGTCGACTAGTACN$_{10}$ GATAT). The second round of PCR (30 cycles) was performed at a higher stringency (annealing temperature of 55°C) by using as the DNA template the PCR products obtained in the first round. The second round of PCR utilized a primer recognizing the insertion element of Tn*cam* (1-L, 5′CTGCCTCCAGAG CCTG) and a primer annealing to the 5′ tag created in the first round (ARB2, 5′GGCCACGCGTCGACTAGTAC). The resulting PCR products were gel purified and then used as a template for DNA sequence analysis, as previously described (37). The *lrhA* and *o405* (accession no. AE000318) genes were cloned into the high-copy pCR-Script vector (Stratagene). DNA encoding each gene was generated by PCR amplification from a chromosomal preparation of the Tn*cam49* mutant. We used a primer annealing within Tn*cam* for *lrhA* (out1-L, CAGGCTCTCCCCGTGGAG) and *o405* (out1-R, CTCCACGGGGAGAGCC TG) in combination with a primer overlapping the 3′ end of either gene (lrhA3′, CTATCGTCCGTCG; o4053′, GGCAGTGAAATTAAG). The PCR products were purified and used for subsequent ligation into the pCR-Script vector according to the manufacturer's recommended procedure [Amp SK(+) cloning kit; Stratagene].

RESULTS

***ompF* growth phase regulation.** In order to identify loci involved in RpoS-dependent repression at *ompF*, a Tn*cam* insertion mutagenesis screen was performed and mutations which increased expression of *ompF′-lacZ* in the presence of the constitutively repressing *ompR107* allele were isolated (36). The expectation was that some of the Lac$^+$ insertions would result from an alleviation of stationary-phase repression and thereby identify regulatory components functioning either upstream or downstream of RpoS. In order to distinguish the mutations that altered RpoS-dependent expression of *ompF′-lacZ* from those affecting other regulatory pathways, each was placed in double-mutant combination with an *rpoS*::*kan* null allele (36), with the expectation that mutations which specifically perturbed RpoS regulation would be nonadditive in comparison to the *rpoS*::*kan* single-mutant phenotype. There were two loci identified, *sprE* and *crl*, which met this specific requirement (36, 37). Here, we describe the characterization of a subset of the mutations which did not behave in clearly additive fashion with *rpoS*::*kan* in this simple double-mutant test. However, these mutations specifically perturbed growth phase regulation of *ompF′-lacZ*, and for this reason we continued to pursue the nature of the stationary-phase defect and its relation to RpoS regulation.

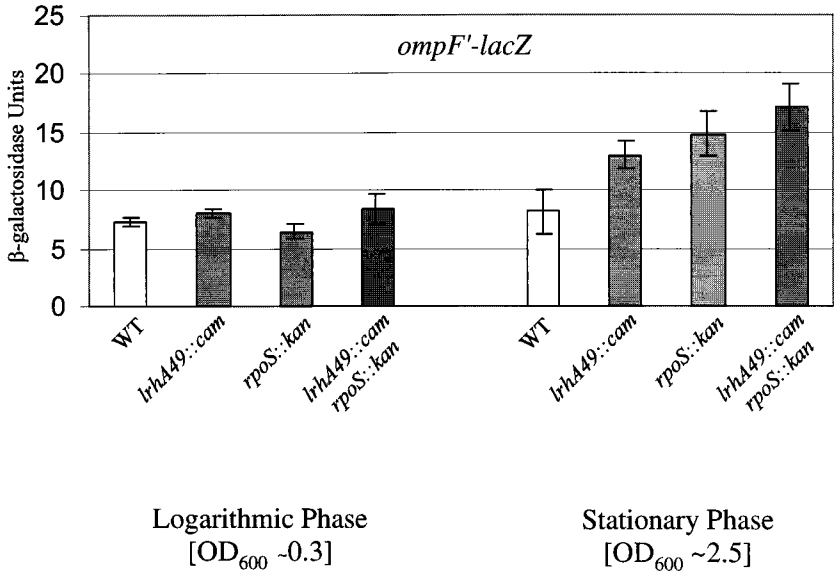

FIG. 1. Effect of *lrhA49*::*cam* on *ompF'-lacZ* expression. Each strain was grown in LB broth at 37°C with aeration overnight and then subcultured at 1:100 into LB broth. To determine logarithmic-phase phenotypes, β-galactosidase activity from the *ompF'-lacZ* gene fusion was assayed when cultures reached an OD_{600} of ~0.3. The same strains were grown in LB broth at 37°C for 24 h to obtain analogous stationary-phase cultures. The wild-type (WT) parent is KEG400 (*ompF'-lacZ*), and the mutant derivatives are KEG403 (*lrhA49*::*cam*), KEG404 (*rpoS*::*kan*), and KEG405 (*lrhA49*::*cam rpoS*::*kan*).

The 52-min linkage group. We found that eight independently isolated Tn*cam* insertions obtained in the screen described above belong within a single linkage group. These mutations were mapped by Hfr crosses to approximately 52 min and were subsequently found to cotransduce with phage P1*vir* at 94% with Δ(*ackA pta*) at 51.8 min (45). In order to avoid confusion, we will refer to a representative mutant from this linkage group as *lrhA49*::*cam* and will justify this nomenclature below.

***lrhA49*::*cam* causes pleiotropic stationary-phase defects.** The transcriptional activity at *ompF* responds to two major signals: medium osmolarity (13) and stationary-phase growth (27, 36). As a first approximation in determining which, if either, regulatory pathway was perturbed, the *ompF'-lacZ* phenotype of each mutant was compared to that of the isogenic parent in logarithmic and stationary phase. We expected that altered osmotic regulation would be detected as a derepression of *ompF'-lacZ* under both growth conditions. In contrast, altered growth phase regulation would be detected as a specific defect in stationary-phase expression of *ompF'-lacZ*, as was previously observed in an *rpoS* null mutant (36).

Each of the mutations in the 52-min linkage group conferred a similar growth phase defect in expression of *ompF'-lacZ*. A representative mutant, *lrhA49*::*cam*, resulted in an approximately twofold derepression of *ompF'-lacZ* expression specifically during stationary-phase growth, similar to what we observed with the *rpoS*::*kan* null allele (Fig. 1). Logarithmic-phase expression, and more specifically osmotic regulation (data not shown), of *ompF'-lacZ* remained unperturbed (Fig. 1). The *lrhA49*::*cam* mutation has no effect on *ompF'-lacZ* expression in an $ompR^+$ background (data not shown). This is not surprising, since *rpoS*::*kan* results in only a modest increase in *ompF'-lacZ* in the presence of $ompR^+$ (36), and it further supports the conclusion that *lrhA49*::*cam* plays no role in modulating the OmpR/EnvZ regulatory pathway. As was alluded to previously, *ompF'-lacZ* expression in the *lrhA49*::*cam rpoS*::*kan* double mutant may be subtly increased in comparison to the single mutants (Fig. 1); however, we suggest that the effects of *lrhA49*::*cam* are largely dependent upon RpoS (see Discussion).

To determine if *lrhA49*::*cam* affected the stationary-phase response in general, we examined the expression of several other genes known to be regulated by RpoS. The *lrhA49*::*cam* mutant was unable to hydrolyze H_2O_2 to the same degree as the wild type when a 30% H_2O_2 solution was dropped onto patched bacteria (determined visually as a bubbling that reflected the amount of O_2 generated). This "fizzing" phenotype depends upon synthesis of the KatE catalase, which is induced by RpoS as bacteria enter stationary phase (31). The stationary-phase expression of *bolA'-lacZ* (21) (Fig. 2) and to a lesser

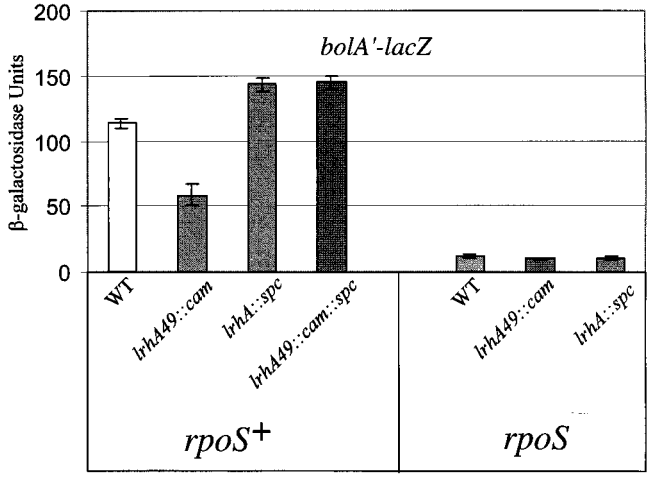

FIG. 2. Effect of *lrhA49*::*cam* on *bolA'-lacZ* expression. Each strain was grown in LB broth at 37°C with aeration, and cells were harvested at 24 h of growth to determine the β-galactosidase activity of the *bolA'-lacZ* strain during stationary phase. The wild-type (WT) parent is ZK916, and its derivatives are KEG445 (*lrhA49*::*cam*), KEG446 (*lrhA*::*spc*), LP867 (*rpoS*::*kan*), KEG448 (*rpoS*::*kan lrhA49*::*cam*), and KEG449 (*rpoS*::*kan lrhA*::*spc*).

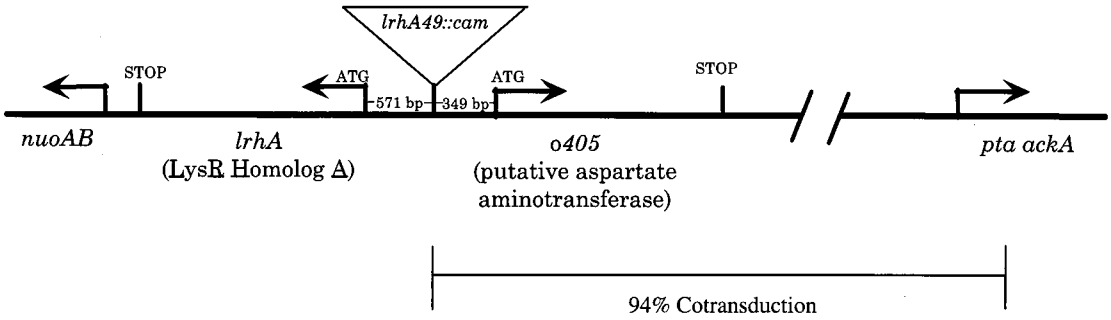

FIG. 3. Diagrammatic representation of *lrhA49*::*cam* physical location. *lrhA49*::*cam* (as well as seven other insertions) was mapped by Hfr and P1*vir* two-factor crosses to approximately 52 min on the *E. coli* chromosome. Sequencing of the *lrhA49*::*cam* insertion junction revealed its location between two open reading frames, *lrhA* and *o405* (accession no. AE000318). Directly downstream of *lrhA* is the *nuoAB* operon encoding the NADH dehydrogenase I enzyme complex.

extent *poxB'-lacZ* (9) (data not shown), which are subject to RpoS-dependent stationary-phase induction, were also decreased in the presence of *lrhA49*::*cam*. These observations indicate that the *lrhA49*::*cam* mutation disrupts the activity of a factor involved globally in growth phase regulation, rather than specifically in *ompF* stationary-phase expression, such that proper development of multiple aspects of stationary-phase physiology is hindered.

***lrhA49*::*cam* results in overexpression of *lrhA*.** Through PCR amplification and DNA sequence analysis of the chromosomal DNA adjacent to the Tn*cam* mutations, we were able to determine their precise insertion junctions. These mutations were found to be inserted at the same site within an intergenic region between two genes of unknown function, suggesting that the insertions most likely alter the expression of one or both of the adjacent genes (Fig. 3). The *lrhA* gene has significant homology to LysR (3), suggesting that it functions as a DNA-binding transcription factor, while the *o405* gene was recognized to possess homology to aspartate aminotransferase (2).

Both genes, *lrhA* and *o405*, were cloned following PCR amplification from the *E. coli* chromosome into the pCR-Script vector such that gene expression would be under the control of the native promoter. Each plasmid was then tested for regulatory effects on *ompF* in KEG400 (MC4100 *ompF'-lacZ ompR107*). The presence of *lrhA* in multicopy increased the expression of *ompF'-lacZ* twofold, phenocopying *lrhA49*::*cam*, while *o405* in multicopy had no effect on *ompF'-lacZ* expression. This result suggests that these Tn*cam* mutations result in either increased or constitutive expression of *lrhA*.

Consistent with the previous observation, the null allele *lrhA*::*spc* (3) was found to have a phenotype opposite that of *lrhA49*::*cam* and resulted in decreased *ompF'-lacZ* expression (data not shown), while expression of *poxB'-lacZ* (data not shown) and *bolA'-lacZ* (Fig. 2) was increased. Further, we determined the *bolA'-lacZ* phenotype of a double mutant in which the *lrhA49*::*cam* insertion was combined in *cis* with the *lrhA*::*spc* null allele. The phenotype of the double mutant was indistinguishable from that of the *lrhA*::*spc* mutant (Fig. 2). Since this *lrhA*::*spc* allele was previously shown to have no effect upon expression of the *nuo* locus (3), these data argue against the possibility that *lrhA49*::*cam* alters stationary-phase regulation through effects on these genes. We conclude from this series of observations that *lrhA49*::*cam* is a gain-of-function mutation that increases *lrhA* expression and that *o405* plays no role in the altered stationary-phase regulation we observe. This justifies the designation *lrhA49*::*cam* for this insertion mutation.

LrhA functions within the RpoS pathway to alter stationary-phase gene expression. As described above, *lrhA* mutations perturb stationary-phase expression of *bolA'-lacZ* (Fig. 2). We were thus able to utilize this transcription fusion to determine the epistatic relationship between *lrhA* and *rpoS*. The *lrhA49*::*cam* allele was introduced into an *rpoS*::*kan* mutant, and the stationary-phase phenotypes of the single and double mutants were determined. We reasoned that if LrhA functions in a growth phase regulatory pathway other than the RpoS pathway, we should observe an additive double-mutant phenotype such that *bolA'-lacZ* expression in the double mutant would be decreased in comparison to either single mutant. However, if LrhA functions within the RpoS pathway, the double mutant should have a phenotype similar to that of either the *rpoS*::*kan* null mutant or the *lrhA49*::*cam* overexpression mutant, depending upon where each gene functions within the RpoS pathway. If *rpoS* functions upstream of *lrhA*, then we should expect the double mutant to have a phenotype similar to *lrhA49*::*cam*. However, if *lrhA* functions upstream of *rpoS*, we should expect the double-mutant phenotype to be identical to that of the *rpoS*::*kan* mutant. What we observed in the double mutant was a nonadditive level of *bolA'-lacZ* expression equivalent to that of the *rpoS*::*kan* single mutant (Fig. 2). We found that the *lrhA*::*spc rpoS*::*kan* double mutant is also phenotypically equivalent to the *rpoS*::*kan* single mutant (Fig. 2), further supporting the conclusion that *rpoS* is epistatic to *lrhA*. From these results, we conclude that LrhA acts upstream of RpoS to down-regulate the RpoS pathway and thus indirectly perturbs stationary-phase expression of *bolA*, and probably *katE*, *poxB*, and *ompF* as well.

***lrhA* mutations perturb growth phase regulation of RpoS posttranslationally.** Since LrhA plays a role in modulating the activity of the RpoS pathway, we wanted to determine whether it functions to vary the levels of RpoS or whether LrhA acts in concert with RpoS to regulate stationary-phase development. Using the *rpoS742'-lacZ* operon fusion (20), we found that transcription of *rpoS* is unperturbed in the presence of *lrhA49*::*cam* (data not shown). In contrast, *lrhA49*::*cam* decreases expression of the *rpoS742'-'lacZ* protein fusion (30) by approximately twofold (Fig. 4), revealing that LrhA functions to regulate RpoS levels posttranscriptionally. The *lrhA*::*spc* mutant has the opposite phenotype, resulting in increased *rpoS742'-'lacZ* expression, and it overrides *lrhA49*::*cam* in the double mutant (Fig. 4A).

Similar regulatory effects are observed when *rpoS742'-'lacZ* expression is assayed in logarithmic phase (Fig. 4A). When *rpoS742'-'lacZ* expression was assayed throughout the growth curve, we found that *lrhA49*::*cam* caused decreased expression

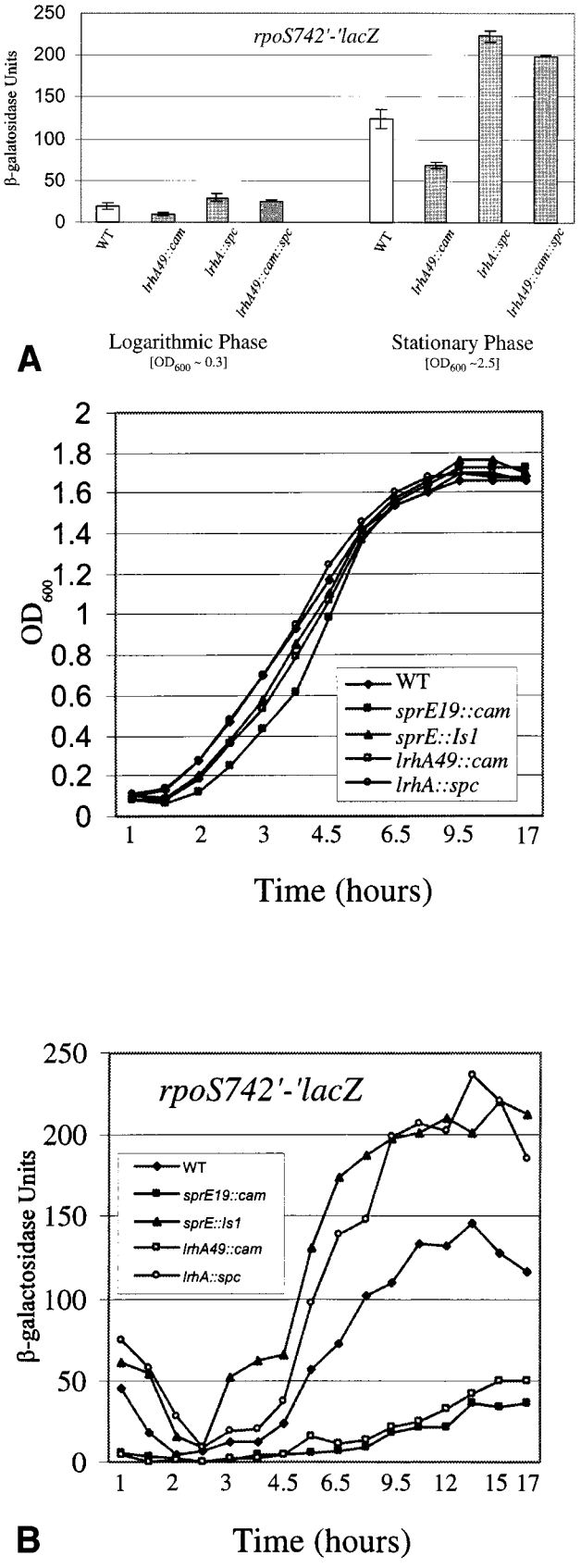

FIG. 4. Effects of *lrhA* mutations on *rpoS742'-'lacZ* expression. (A) Strains were grown in LB broth at 37°C with aeration overnight and then subcultured at 1:100 into LB broth. To determine logarithmic-phase phenotypes, β-galactosidase activity from the *rpoS742'-'lacZ* gene fusion was assayed when cultures reached an OD_{600} of ~0.3. The same strains were grown in LB broth at 37°C for 24 h to obtain analogous stationary-phase cultures. The wild-type (WT) parent is RO91 (*rpoS742'-'lacZ*), and its derivatives are KEG408 (*lrhA49*::*cam*), KEG409 (*lrhA*::*spc*), and KEG410 (*lrhA49*::*cam*::*spc*). (B) Strains were grown in LB broth at 37°C with aeration overnight and then subcultured at 1:100 into LB broth. Aliquots of each culture were taken at the indicated time points, and both the OD_{600} and β-galactosidase activity the samples were determined. The wild-type (WT) parent is RO91, and its mutant derivates are indicated in the figure.

at all time points and allowed only weak induction as bacteria entered stationary phase (Fig. 4B). We also observed greater induction of *rpoS742'-'lacZ* during mid-logarithmic-phase growth in the *lrhA*::*spc* mutant (Fig. 4B).

These *lrhA* growth curve phenotypes mimic what was observed with analogous overexpression and null alleles of *sprE* (36) (Fig. 4B) and suggested that LrhA could also be involved in growth phase regulation of RpoS accumulation. Therefore, we determined the steady-state level of RpoS during stationary phase by Western blot analysis in the *lrhA* mutant backgrounds (Fig. 5). In particular, the *lrhA49*::*cam* mutation results in dramatically decreased RpoS levels such that the protein is barely detectable. This inability to accumulate RpoS during the transition into stationary phase in the *lrhA49*::*cam* mutant has clear effects on the ability of these bacteria to induce genes such as *bolA*, *katE*, and *poxB*, which are important for viability during stationary phase (19, 24).

It has been proposed that modulation of the rate of RpoS degradation is the major control point for determining the amount of RpoS that accumulates throughout the growth curve (46). During logarithmic growth, RpoS is maintained at low levels due to degradation mediated by SprE and the ClpXP protease (29, 36, 40), and the *rpoS742'-'lacZ* protein fusion is susceptible to this regulated proteolysis (30). In order to determine whether LrhA acts within the SprE/ClpXP pathway, we performed tests of epistasis between alleles of *lrhA* and the null alleles *sprE*::*IS1* and *clpX*::*kan*. We found that the presence of either the *lrhA49*::*cam* or the *lrhA*::*spc* allele had no phenotypic effect on *rpoS742'-'lacZ* in the absence of SprE or ClpX (Fig. 6). Therefore, *lrhA* functions to promote the degradation of RpoS through the genetically defined SprE/ClpXP pathway. Furthermore, LrhA must act upstream of the other known components of this pathway since the *lrhA49*::*cam* overexpression allele is phenotypically silent in the double mutants.

LrhA does not regulate synthesis of ClpX, ClpP, or SprE. Since LrhA has homology to the LysR-like family of transcription factors (3), possessing a canonical helix-turn-helix DNA-binding domain, we wanted to determine whether LrhA functions to regulate the expression of *sprE*, *clpX*, or *clpP* and thereby to affect RpoS accumulation. We were able to genetically determine whether LrhA plays a regulatory role at the *sprE* promoter by utilizing the allele *sprE19*::*cam* (36). In *sprE19*::*cam* strains, *sprE* expression is uncoupled from its native promoter and is constitutively expressed independently of the native promoter's activity. This Tn*cam* is inserted 22 bp upstream of the start codon of *sprE* and has been found by Western blot analysis to result in constitutively increased levels of SprE (data not shown). There is a promoter within the transposon oriented towards *sprE* that is most likely responsible for stimulating this constitutive overexpression. We combined the *sprE19*::*cam* allele with *lrhA*::*spc* and determined the phenotype of the double mutant by assaying *rpoS742'-'lacZ* expression. Any effect of LrhA at the native *sprE* promoter should be blocked by the *sprE19*::*cam* mutation. However, we observed an increase in *rpoS742'-'lacZ* expression in the dou-

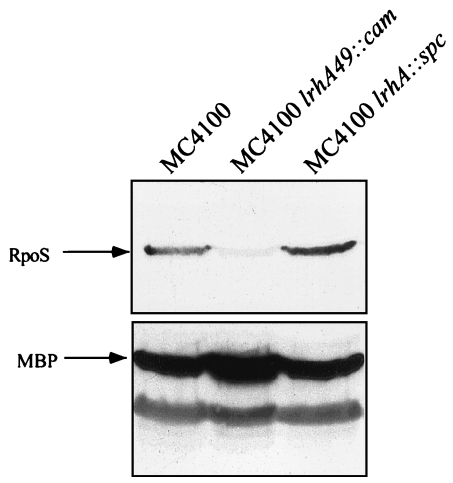

FIG. 5. Immunoblot analysis of σ^S. RpoS is indicated by an arrow in MC4100 and its *lrhA* mutant derivatives. Each strain was grown in LB broth at 37°C with aeration, and cells were harvested after 24 h of growth at an OD_{600} of ~2.5 to obtain a whole-cell lysate, as described in Materials and Methods. After immunoblot analysis with anti-RpoS antibodies, the blot was stripped and reprobed with anti-MBP antibody to control for loading.

ble mutant (Fig. 7), suggesting that LrhA does not function to regulate the activity of the native *sprE* promoter. Moreover, *lrhA* mutants have no effect upon stationary-phase SprE levels, as determined by Western blot analysis (data not shown). Thus, we conclude that LrhA does not regulate SprE synthesis.

We assayed for potential regulation of *clpX* and *clpP* by determining protein levels through Western blot analysis from both logarithmic- and stationary-phase cultures. We found no differences in the level of ClpX or ClpP in either stationary phase (Fig. 8) or logarithmic phase (data not shown) when comparing the *lrhA* mutants to the isogenic parental strain MC4100. The synthesis of neither SprE nor the ClpXP protease is altered in *lrhA* mutants. Since it was demonstrated previously that *clpX* and *clpP* are epistatic to *sprE* (36), and our epistasis data place *lrhA* function upstream of *sprE*; these final observations imply that LrhA acts to modulate the activity of the response regulator SprE.

DISCUSSION

To better understand the pathways which regulate *ompF* expression in response to environmental and growth phase signals, Tn*cam* insertion mutagenesis was performed (36). This screen has successfully identified a number of novel components involved in stationary-phase-dependent RpoS repression at *ompF*. All of the components characterized thus far function to regulate RpoS accumulation and/or activity (36, 37). There are no known downstream effectors of RpoS regulation at *ompF*, suggesting that RpoS may repress expression at *ompF* directly. However, the mechanism of repression remains to be clarified.

We have found that LrhA is another component of the *ompF* growth phase regulatory pathway and that it is involved in modulating stationary-phase-dependent RpoS accumulation. LrhA functions to modulate RpoS levels by promoting rapid degradation during logarithmic growth (Fig. 9). This conclusion is based partially on the observation that the *lrhA49*::*cam* overexpression allele allows very little stationary-phase accumulation of RpoS, while in contrast the *lrhA*::*spc* null allele allows greater RpoS accumulation during logarithmic growth. More telling is the observation that LrhA-dependent inhibition of RpoS accumulation absolutely requires the response regulator SprE and the ClpXP protease, placing LrhA upstream of SprE within this genetic pathway. Previous studies have determined that SprE acts upstream of ClpXP (36) in a substrate-specific manner to target RpoS for degradation (46). Since LrhA does not control the synthesis of SprE, we further suggest that LrhA promotes RpoS degradation by modulating the activity of SprE.

SprE has been identified as a member of the family of two-component response regulators based on homology (4). As such, its activity is most likely modulated via phosphorylation at a conserved aspartic acid residue (43). However, a cognate sensor histidine kinase has yet to be identified for SprE, and so it remains unclear how SprE activity is growth phase modulated and to what specific signal(s) it responds. As has been

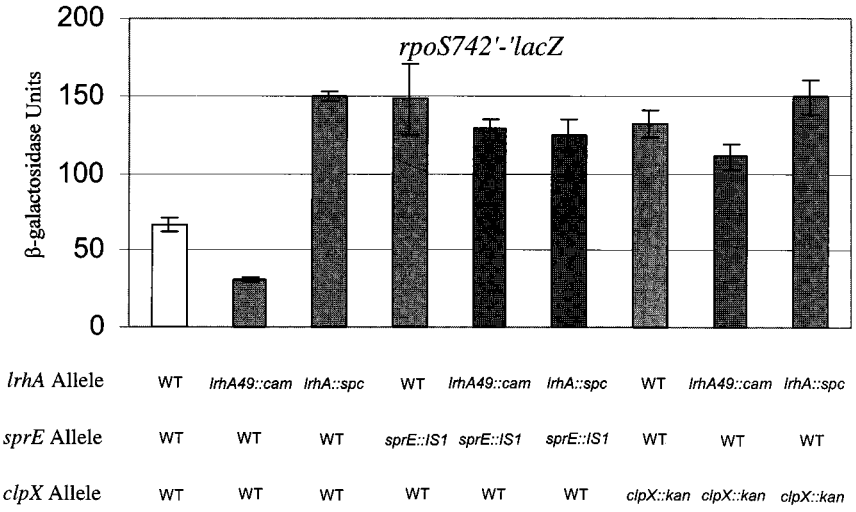

FIG. 6. Epistasis test between *lrhA* and *sprE*::*Is1* or *clpX*::*kan*. Each strain was grown in LB broth at 37°C with aeration, and cells were harvested after 24 h of growth at an OD_{600} of ~2.5 to determine the stationary-phase *rpoS742'-'lacZ* phenotype. All strains are derived from RO91 (*rpoS742'-'lacZ*) and contain the indicated mutation(s). WT, wild type.

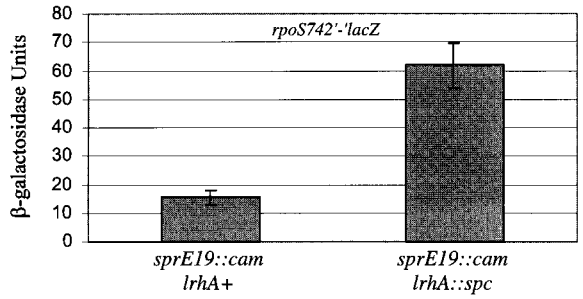

FIG. 7. Epistasis test between *lrhA*::*spc* and *sprE19*::*cam*. Each strain was grown in LB broth at 37°C with aeration, and cells were harvested after 24 h of growth at an OD_{600} of ~2.5 to determine the stationary-phase *rpoS742'-'lacZ* phenotype. All strains are derived from LP801 (*rpoS742'-'lacZ sprE19*::*cam*) and contain the indicated *lrhA* allele.

observed with other response regulators, SprE can be phosphorylated in vitro by acetyl phosphate (5). This phosphorylation can be further detected in vivo as a SprE-dependent increase in *rpoS742'-'lacZ* expression in a Δ(*pta ackA*) mutant (5). However, as there is still stationary-phase induction of RpoS in the Δ(*pta ackA*) mutant, there must be an additional signal(s) which mediates growth phase regulation of SprE activity.

We propose that LrhA and acetyl phosphate modulate SprE activity independently. This is based upon initial experiments performed to elucidate LrhA function at *ompF*. In *envZ*::*cam* null strains, the orphaned response regulator OmpR is dependent upon phosphorylation by acetyl phosphate for DNA-binding activity at *ompF* (17), thereby allowing us to use OmpR-dependent activation at *ompF* to monitor acetyl phosphate levels. In an *envZ*::*cam rpoS*::*kan* background, we were able to determine whether LrhA had an effect upon *ompF'-lacZ* in the absence of SprE-dependent stationary-phase regulation. We observed that introduction of the *lrhA*::*spc* null mutant had no effect upon the phosphorylation level of OmpR; i.e., *ompF'-lacZ* expression remained unchanged (data not shown). If

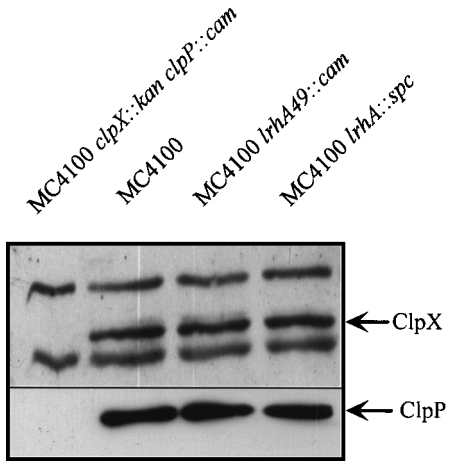

FIG. 8. Immunoblot analysis of ClpX and ClpP. The ClpX and ClpP proteins in MC4100 and the indicated *lrhA* derivatives (the *clpX*::*kan clpP*::*cam* mutant is included as a control) are indicated by arrows. Each strain was grown in LB broth at 37°C with aeration, and cells were harvested after 24 h of growth at an OD_{600} of ~2.5 to obtain a whole-cell lysate, as described in Materials and Methods. After immunoblot analysis with anti-ClpX antibodies, the blot was stripped and reprobed with anti-ClpP antibodies (the same result was obtained regardless of whether the blot had been previously probed and stripped [data not shown]).

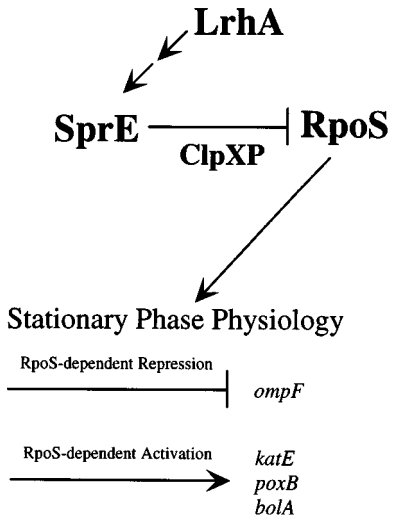

FIG. 9. Genetic pathway required for growth phase-dependent regulation of RpoS degradation. LrhA is the most upstream component and somehow functions to modulate SprE activity. The phosphorylated form of SprE promotes degradation of RpoS by the ClpXP protease, probably in response to nutrient limitation. Then, RpoS, functioning as a sigma factor, is able to recruit RNAP to a subset of stationary-phase-inducible promoters and thereby acts as a global regulatory protein required for the physiological adaptations characteristic of stationary-phase bacteria.

LrhA was involved in regulating acetyl phosphate levels, we would expect a decrease in *ompF'-lacZ* levels due to indirect effects upon OmpR activity. Instead, our observation suggests that LrhA regulates RpoS levels independently of acetyl phosphate and that there are probably multiple inputs which modulate SprE activity.

LrhA, as a putative DNA-binding transcription factor (3), is likely to regulate SprE activity indirectly. Perhaps LrhA regulates expression of an as-yet-unidentified cognate sensor histidine kinase. Alternatively, LrhA might regulate the expression of metabolic enzymes, as is observed with many LysR family members (39), the activity of which might in turn be sensed by SprE either indirectly, through a cognate histidine kinase sensor, or directly, through a small molecule phosphodonor or a phosphorylated metabolic enzyme. The direct regulatory targets of LrhA are unknown, but identification of these genes will provide an important link in our understanding of how SprE activity is modulated. Further characterization of LrhA function may also provide a means toward understanding how *E. coli* senses the growth rate in order to regulate RpoS appropriately.

LrhA was originally identified as a putative DNA-binding transcription factor based on homology to the LysR family of proteins. This LysR homology suggests that LrhA activity may be modulated via binding of a small molecule inducer (32). Typically, LysR homologs regulate expression of genes functioning within a metabolic pathway, and their activity is in turn modulated through binding of a metabolic intermediate, which is either utilized or produced by the regulated pathway. We do not yet understand how LrhA activity is regulated; i.e., the molecular nature of the coinducer and how its levels fluctuate are unknown. It may be that coinducer synthesis changes in response to the bacterial growth rate, and in this way LrhA would be able to modulate SprE activity, and thus RpoS accumulation, according to nutrient availability.

As noted above, additivity tests with *lrhA49*::*cam* and *rpoS* do not yield clear answers about the requirement for RpoS in

the stationary-phase derepression of *ompF*. We suspect that this problem relates to nonspecific phenotypes caused by LrhA overexpression. The stress caused by excess LrhA probably affects *ompF* expression through an indirect mechanism that is unrelated to normal cellular function.

LrhA is most closely related to the PecT regulator of pectinolysis in *Erwinia chrysanthemi* (43) and the HexA regulator of pectinolysis and motility in *Erwinia carotovora* (14). *E. chrysanthemi* is a phytopathogenic enterobacterium that requires induction and secretion of a group of pectinolytic enzymes in order to cause soft rot disease in a variety of plants (18). The pectinase genes are regulated at the level of transcription in response to a variety of signals, including the presence of pectin and plant extracts, stationary-phase growth, osmolarity, low temperature, oxygen and nitrogen availability, and iron limitation. *pecT* was found in a screen designed to identify genes involved in regulating pectate lyase synthesis and was shown to function as a transcriptional repressor of five pectate lyase genes (43). As with LrhA, it is not known what environmental conditions regulate PecT activity.

In a subsequent study of pectinolysis regulation, a Tn*5* insertion mutation which decreased the expression of a number of pectate lyases was isolated (8). This Tn*5* was inserted within the intragenic region between *pecT* and the adjacent putative aspartate aminotransferase, 579 bp upstream of the *pecT* gene, in a manner highly analogous to the *lrhA49*::*cam* group of mutants at the *lrhA* locus described herein. The adjacent putative aspartate aminotransferase was shown to play no role in the Tn*5* mutant phenotype or in pectate lyase regulation. Instead the Tn*5* caused increased transcription at *pecT* by preventing PecT negative autoregulation. Since LrhA is 95% similar to PecT in the first 120 amino acids, which contain the putative DNA-binding domain, and since *lrhA49*::*cam* phenocopies *lrhA* overexpression, we infer that LrhA functions as a DNA-binding protein that negatively autoregulates its own expression. Furthermore, we would suggest, based on our observations, that perhaps PecT regulates the *E. chrysanthemi* pectate lyases in a growth phase-dependent manner through regulatory effects upon RpoS.

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