except that lysis buffer lacked $CaCl_2$ or $MgCl_2$. Rabbit polyclonal antiserum, prepared against polyomavirus ST but recognizing all three tumor antigens because of their shared NH_2-terminal sequence identity (*18*), was used to immunoprecipitate MT.

7. Microsequencing was performed on proteins purified from lysates that were shown previously by tryptic peptide analysis to be identical to the 27- and 29-kD MT-associated proteins (*5*).
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9. Polyclonal rabbit serum was raised against the peptide ELVQKAKLAEQA synthesized on a polylysine core (MAP resin). The identical sequence is found in several 14-3-3 family members, including the zeta form, and less related versions are found in many other family members, including the epsilon form (*19*).
10. Subconfluent NIH 3T3 cells (5×10^6) stably expressing wild-type MT from an integrated retroviral vector (*20*) were washed and lysed as in (*6*). Polyclonal rabbit antiserum, and preimmune serum from the same animal were used to immunoprecipitate MT (*18*), and the immunoprecipitates were analyzed by 2D gel electrophoresis (*14*). Proteins were transferred electrophoretically and immunoblotted (*21*) with the antibody described in (*9*).
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13. Subconfluent NIH 3T3 cells (2×10^6) stably expressing polyomavirus ST, MT, or $pp60^{v\text{-}Src}$ from an integrated retroviral vector and Sf9 insect cells (2×10^6) infected with wild-type or MT-expressing baculovirus were lysed as described in (*6*). Immunoprecipitates were prepared from cell lysates with polyclonal rabbit antitumor antigen serum (*18*) and with control serum and tested for their ability to activate the ADP-ribosyltransferase activity of exoenzyme S of *P. aeruginosa* as in (*12*), using 3 μl of immune complexes and the artificial substrate soybean trypsin inhibitor (100 μg/ml; Sigma) in a final reaction volume of 10 μl. The assay was performed at 23°C for 30 min. [^{32}P]ADP-ribose incorporation into substrate was verified by analysis on gels. In the absence of the immune complexes, exoenzyme S had no detectable ADP-ribosyltransferase activity. Background values obtained with preimmune sera were subtracted from each sample.
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Sensing Starvation: A Homoserine Lactone–Dependent Signaling Pathway in *Escherichia coli*

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When nutrients become limiting, many bacteria differentiate and become resistant to environmental stresses. For *Escherichia coli*, this process is mediated by the σ^s subunit of RNA polymerase. Expression of σ^s was induced by homoserine lactone, a metabolite synthesized from intermediates in threonine biosynthesis. Homoserine lactone–dependent synthesis of σ^s was prevented by overexpression of a newly identified protein, RspA. The function of homoserine lactone derivatives in many cell density–dependent phenomena and the similarity of RspA to a *Streptomyces ambofaciens* protein suggest that synthesis of homoserine lactone may be a general signal of starvation.

Bacteria lead a "feast or famine" existence, interchanging short periods of rapid growth with prolonged periods of starvation. To survive starvation, bacteria develop stress resistances before nutrients are exhausted (*1*). These developmental changes require the induction of specific genes at the onset of starvation, a process that in *E. coli* is in part regulated by the induction of a stationary phase–specific sigma factor of RNA polymerase, termed σ^s or σ^{38}. The mechanism by which bacteria sense starvation and how this signal is transduced to induce σ^s synthesis is only poorly understood.

The σ^s protein is encoded by the *rpoS* gene and regulates expression of at least 30 genes, some of which influence osmoprotection (*osmB*, *osmE*, *otsAB*, *treA*), cell morphology (*bolA*), or general stress resistance (*katE*, *xth*, *dps*, *appA*, *mccC*) (*1*). Transcription of *rpoS* increases at the onset of stationary phase (*2*), and the σ^s protein also appears at that time (*3*, *4*). To understand further the mechanism by which bacteria sense starvation and respond by inducing σ^s activity, we tested whether any *E. coli* genes, when present in high copy number, could alter the expression of a σ^s-dependent *bolA::lacZ* gene fusion (*5*). We found one chromosomal locus that in high copy completely repressed expression of this fusion (Fig. 1A) (*6*).

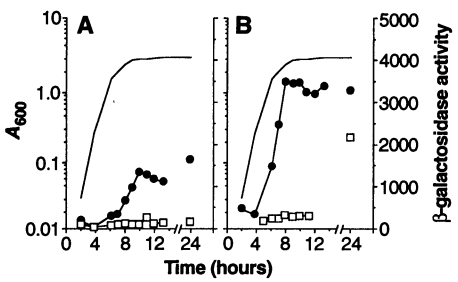

Fig. 1. Repression of σ^s-dependent gene expression by pSPER1. *Escherichia coli* VIP36 (*bolA::lacZ*) (**A**) and *E. coli* HS143 (*rpoS::lacZ*) (**B**) were transformed with pSPER1 (open squares) and pUC19 (closed circles), and β-galactosidase activities (Miller units) were determined during growth and stationary phase (line with no symbols). Cultures were grown in LB medium at 30°C.

Strains harboring this clone (pSPER1) also failed to induce *rpoS* expression at the onset of stationary phase (Fig. 1B). The chromosomal insert present in pSPER1 was located at minute 35.6 of the *E. coli* chromosome (*7*). The nucleotide sequence of the region identified two adjacent genes, designated *rspA* and *rspB*, whose products were overproduced as determined by SDS–polyacrylamide gel electrophoresis (PAGE) (*8*). Repression of *rpoS* expression was correlated with the overproduction of RspA (Fig. 2A).

The amino acid sequence of RspA is similar (*9*) to that of Spa2, a protein from *Streptomyces ambofaciens* (Fig. 2B). Strains in which the locus encoding Spa2 is amplified are deficient in starvation-induced pigmentation (*10*). RspA is also similar to two catabolic enzymes from *Pseudomonas putida*—mandelate racemase and chloromuconate cycloisomerase (*11*)—that have nearly identical three-dimensional structures and share active site residues that are conserved in RspA (Fig. 2B) (*11*).

The similarity of the RspA sequence to that of catabolic enzymes led us to hypothesize that the effect of RspA on *rpoS* transcription could result from degradation of a metabolite that signals starvation (*12*). The similarity of RspA with a lactonizing enzyme (chloromuconate cycloisomerase) suggested that such a metabolite might be a lactone. Homoserine lactone (HSL) derivatives act as inducers in several cell density–dependent systems including bioluminescence, Ti plasmid transfer, and synthesis of exoenzymes and antibiotics (*13*).

Although HSL derivatives are known to act as inducers of diverse phenomena, the biosynthetic pathway of HSL has not been described. We reasoned that possible precursors for the formation of HSL might be homoserine (HS) and homoserine phosphate (HSP)—intermediates in the threonine biosynthetic pathway (Fig. 3). If these

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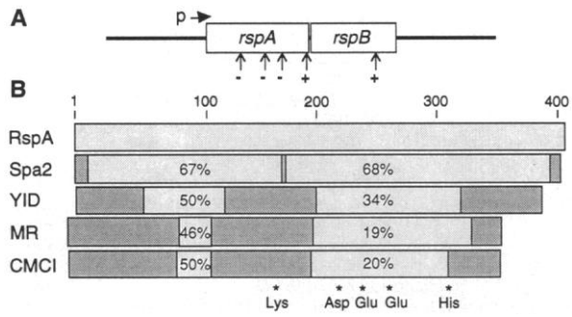

Fig. 2. Genetic analysis of the pSPER1 insert. (**A**) Nucleotide sequencing of the *rpoS*-repressing locus identified two genes, *rspA* and *rspB*. These genes form an operon because transposon insertions in *rspA* abolish *rspB* expression. Arrows indicate sites of transposon insertions and the ability of these plasmids to repress *bolA::lacZ* expression as determined by colony phenotypes on lactose indicator medium (+, represses; −, does not repress). In addition, a 28–amino acid COOH-terminal RspA deletion prevented *bolA::lacZ* repression, although overproduction was not affected. The transcriptional promoter is represented by "p." (**B**) Similarity of the primary structure of RspA to that of Spa2 from *Streptomyces ambofaciens*, the COOH-terminal end of a protein from *E. coli* (YID) (*28*), and mandelate racemase (MR) and chloromuconate cycloisomerase (CMCI) from *Pseudomonas putida* [numbers indicate the percentage of identical amino acids in the given stretches (*9*)]. The asterisks mark conserved residues in MR and CMCI important for catalysis (*11*).

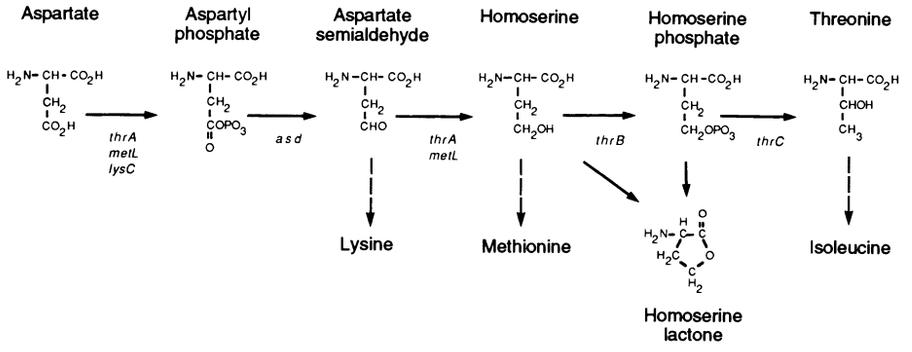

Fig. 3. Biosynthesis of the aspartate group of amino acids. Aspartate is the precursor for the biosynthesis of lysine, methionine, threonine, and isoleucine. The first reaction in this pathway can be catalyzed by three enzymes and is controlled by the three amino acid end products. Homoserine is at the branch point that leads to either methionine or threonine, and its fate is under strict regulatory control of methionine. We propose that homoserine or homoserine phosphate (or both) are precursors of homoserine lactone.

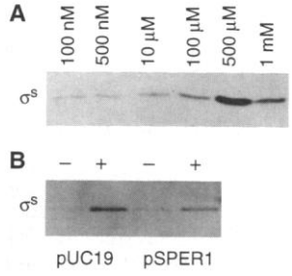

Fig. 4. Effect of homoserine lactone on σ^s expression. The amount of σ^s was analyzed in protein immunoblots with monoclonal antibodies to σ^s (*17*). (**A**) *Escherichia coli* ZK1470 (*thrA metL lysC*) was grown for 16 hours at 30°C in M63-glucose medium supplemented with threonine, methionine, lysine, and the indicated amounts of HSL. Equal volumes of the cultures were loaded and separated by SDS-PAGE (12.5% gel). (**B**) Cultures of *E. coli* ZK1470 (*thrA metL lysC*) carrying pUC19 or pSPER1 were grown at 30°C for 16 hours in M63-glucose medium supplemented with threonine, methionine, and lysine in the presence or absence of 1 mM HSL. Equal amounts of cells were loaded and separated by SDS-PAGE (12.5% gel).

intermediates serve as precursors of HSL and if HSL enhances expression of *rpoS*, then mutants blocked in HS and HSP synthesis would be predicted to be defective in *rpoS* expression. We therefore obtained *E. coli* mutants blocked in every step of threonine biosynthesis (*14*). Initial characterization of the σ^s phenotype of these strains was done with a qualitative plate assay for the expression of the σ^s-dependent catalase HPII (*15*). The strains were grown on minimal medium containing glucose and the appropriate amino acids. Under these conditions, the *thr*C mutant has normal catalase HPII activity. However, no catalase activity was observed in mutants blocked at steps before the synthesis of homoserine; the *thrB* mutant showed low catalase activity. Catalase activity could be restored when HSL or HS (either at 1.2 mM) was included in the growth medium (*16*). These results suggested that HSL (or metabolites derived from it) induced *rpoS* expression and that HSL can be synthesized from HS or HSP (or both).

The effect of HSL concentration on the abundance of σ^s was determined directly by protein immunoblot analysis with monoclonal antibodies to σ^s (*17*). The *thrA metL lysC* triple mutant blocked in the first step of the pathway (Fig. 3) contained very low amounts of σ^s when grown in minimal medium in the absence of HSL. Addition of HSL resulted in an increase in the abundance of σ^s (Fig. 4A), consistent with a role of HSL in regulating the expression of *rpoS* (*18*). Stimulation of σ^s accumulation by HSL was partially blocked by the presence of pSPER1 (Fig. 4B).

In bioluminescent *Vibrio fischeri* an acylated HSL derivative, 3-ketohexanoyl-HSL [known as autoinducer (AI)], is produced by LuxI and subsequently activates LuxR to transcribe the *lux* operon (*19*). *Escherichia coli* strains that contain the *lux* genes are luminescent and this phenotype does not depend on σ^s (*20*). Overproduction of RspA repressed this bioluminescence, indicating that RspA can interfere with a second HSL-related signaling pathway (Fig. 5, A and B). Protein sequence database searches have identified SdiA as an *E. coli* LuxR homolog (*21*); however, an *sdiA* mutant still expresses normal amounts of σ^s-dependent HPII catalase.

The *lux* operon was dissected to deter-

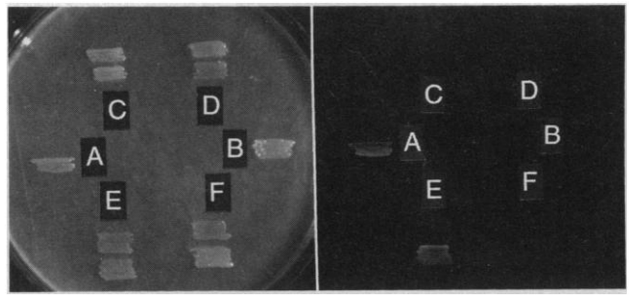

Fig. 5. Repression of bioluminescence by overexpression of RspA. *Escherichia coli* VIP36 was transformed with plasmids that contained all or part of the *Vibrio fischeri lux* system. Cell growth is shown on the left, bioluminescence on the right. (A) *Escherichia coli* VIP36 with a plasmid containing all the *lux* genes (pNL121) and pUC19. (B) *Escherichia coli* VIP36 containing pNL121 and pSPER1. Strains with pAK017 make autoinducer, lack the luciferase genes (AI^+, Luc^-), and can induce luminescence in adjacent strains that carry pAK211, which cannot synthesize AI but contain the luciferase genes (AI^-, Luc^+) (*22*). In the pairs shown in (C) through (F), the top strain has pAK017 and the bottom strain has pAK211 and, in addition (top/bottom), pSPER1/pSPER1 (C), pSPER1/pUC19 (D), pUC19/pUC19 (E), and pUC19/pSPER1 (F).

mine whether RspA acts before or after AI synthesis (Fig. 5) (*22*). Strains that produced and released AI were grown adjacent to strains unable to make AI but which could respond to the signal by inducing the genes encoding luciferase. Bioluminescence was repressed when RspA was overproduced in either strain, presumably because AI was inactivated before it diffused out of the producer cell or as soon as it entered the recipient (*23*). These results indicate that RspA acts after AI synthesis.

HSL appears to be a key signal for inducing transcription of *rpoS*. We propose the following model to explain HSL accumulation at the onset of stationary phase. As cells exhaust carbon and energy sources, imbalances occur in amino acid biosynthetic pathways (*24*). This results in empty acceptor sites in ribosomes, increasing guanosine tetraphosphate synthesis and thus stimulating the expression of threonine biosynthetic enzymes (*25*). Increased threonine levels, coupled with slow translation, cause feedback inhibition, and HS and HSP accumulate. These intermediates could be bound by tRNA synthetases and the proofreading function of these enzymes would produce HSL, a reaction known to occur in vitro (*26*). The finding that a mutant unable to make guanosine tetraphosphate does not induce *rpoS* in Luria-Bertani (LB) medium is consistent with this proposal (*4*).

Bioluminescence and other previously known HSL-dependent regulatory systems are expressed at high cell density (*13, 19*). Under typical laboratory conditions, starvation also occurs at high cell densities. However, σ^s expression occurs equally well when *E. coli* is starved at 10^7 cells per milliliter or 5×10^9 cells per milliliter (*27*). It is possible that HSL constitutes an intracellular signal that accumulates in starved cells regardless of cell density. The increased stress resistance of starved cells is essential for the survival of individual cells and therefore should be independent of the density of the culture. At high cell densities this signal molecule can be acylated to allow diffusion across membranes, thereby converting it into an extracellular signal that benefits the population when high concentrations of cells are present.

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5. A Hind III library in pUC19 of total *E. coli* ZK126 DNA was constructed and introduced into *E. coli* VIP36 [D. E. Bohannon *et al.*, *J. Bacteriol.* **173**, 4482 (1991)], which contains *bolA::lacZ*. Transformants were selected on McConkey-lactose plates containing ampicillin (150 μg/ml) on which the parent strain appears red. Plasmids were isolated from white colonies and designated pSPER (for stationary phase expression regulation). Three plasmid classes were found to reduce expression of *bolA::lacZ*. The inserts in two of these mapped to previously described genes: *ftsA* [A. C. Robinson *et al.*, *J. Bacteriol.* **160**, 546 (1984)] and a gene located between *iclR* and *aceK* [A. Galinier *et al.*, *Gene* **97**, 149 (1991)]. The other plasmid was pSPER1.
6. Expression of other σ^s-dependent genes (*mccC, treA, osmB, osmE*, and *katE*) was also repressed by pSPER1. A σ^s-independent *secA::lacZ* fusion, however, was not affected by pSPER1.
7. The plasmid pSPER1 contains a 6.4-kb Hind III insert. The plasmid was labeled with a nonradioactive DNA labeling kit (Boehringer Mannheim) and used to probe the ordered phage library of the *E. coli* chromosome [Y. Kohara, K. Akiyama, K. Isono, *Cell* **50**, 495 (1987)]. A set of mini-γδ insertion mutants [C. Berg *et al.*, *Gene* **113**, 9 (1992)] was generated to localize and sequence the repressing locus. The nucleotide sequence was determined with the Sequenase kit (U.S. Biochemical).
8. Total proteins of different subclones and mini-γδ insertions in *E. coli* VIP36 were separated by SDS-PAGE and revealed the overproduction (>20-fold) of proteins of 43 and 37 kD in strains carrying either pSPER1 or a 4.4-kb Bam HI subclone. Both proteins were also overproduced in a *rpoS* mutant background. Bands of the overproduced proteins were excised from a blot of such a gel on polyvinylidene difluoride (PVDF) membrane and subjected to NH_2-terminal amino acid sequencing. The determined NH_2-terminal sequences were identical to the predicted NH_2-terminal sequences of two open reading frames of 404 and 340 amino acids, respectively, which are separated by a 14-bp intergenic region. These genes were designated *rspA* and *rspB* (for regulatory in stationary phase). The GenBank accession number for the *rspA* and *rspB* nucleotide sequence is L31628.
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28. The gene *yid* encodes a 587–amino acid protein of which the COOH-terminal 384 residues are homologous to RspA. *yid* was subcloned into pBluescript-KS, and this clone was unable to repress σ^s-dependent catalase activity even though overexpression of the protein was confirmed (G. Mrachko, G. Huisman, P. Babbitt, J. Gerlt, R. Kolter, unpublished results).

29. We thank A. Link for performing NH_2-terminal protein sequencing; L. Nguyen and R. Burgess for providing antibody to σ^s; H. Schellhorn, A. Kuo, P. Dunlap, B. Bachmann, J. Beckwith, C. Gutierrez, and L. Rothfield for strains and plasmids; P. Babbitt, J. Gerlt, G. Kenyon, G. Petsko, A. Kuo, M. Bollinger, H. Paulus, and H. Goodrich-Blair for helpful discussions; and L. Sonenshein and R. D'Ari for critically reading the manuscript. Supported by a grant from NSF (MCB-9207323) to R.K. R.K. was the recipient of an American Cancer Society Faculty Research Award.

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