Isolation of a Carbon Starvation Regulatory Mutant in a Marine *Vibrio* Strain

JÖRGEN ÖSTLING,^{1,2} KLAS FLÄRDH,^{1†} AND STAFFAN KJELLEBERG^{2*}

Department of General and Marine Microbiology, Göteborg University, Göteborg, Sweden,¹ and School of Microbiology and Immunology, University of New South Wales, Sydney, Australia²

Received 1 May 1995/Accepted 28 September 1995

A carbon starvation-responding *lac* fusion of the marine *Vibrio* sp. strain S14 was used as a reporter strain in order to identify genes critical in the regulation of the carbon starvation response. Interestingly, sequence data together with an altered phenotype with respect to the accumulation of guanosine 3',5'-bispyrophosphate (ppGpp) imply that one of the genes (*csrS*) identified by this approach is an *Escherichia coli* *spoT* equivalent. Complementary data suggest that the function encoded by the *csrS* gene is essential for the successful development of starvation and stress resistance.

Natural bacterial habitats are subject to heterogeneous conditions both temporally and spatially. One of the most impressive features of microorganisms is their ability to adapt to such environmental challenges. Bacterial cells that encounter changes in concentrations of a particular nutrient usually compensate their metabolic activities by altering the expression of individual operons. However, total energy depletion is followed by a dramatic reorganization of the cell interior which, in the case of many differentiating bacteria, leads to the formation of metabolically inactive and stress-resistant spores (7, 34). Many nondifferentiating bacteria respond to energy depletion in a similar, albeit morphologically less pronounced, fashion by formation of stress-resistant ultramicrocells. This cell differentiation-like response has been studied on a molecular level for a number of nondifferentiating bacterial species (12, 24, 35, 37, 41), and evidence accumulated over recent years clearly indicates that this process is a highly ordered sequence of intracellular events requiring the concerted regulation of several operons (11, 13, 21, 22, 26, 27). Carbon starvation has previously been shown to be a determinant for the successful formation of ultramicrocells and development of various stress resistances in the marine organism *Vibrio* sp. strain S14 (18, 30). In order to identify the cellular components involved in the regulation of this response, mutants potentially impaired in regulation of the carbon starvation response were isolated.

Generation of a carbon starvation regulatory mutant. The previously described *Vibrio* sp. strain S141 *csi-5::MudI(lac)* fusion strain (J105) (40) was used as a recipient for a secondary transposon [mini-Tn10(Km)], and the mutants were then screened for a constitutive LacZ phenotype (Table 1). Colonies of strain J105 are white during growth on low-glucose X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) minimal-medium plates (40) but become blue when the colony size ceases to increase as a result of glucose depletion. Overnight cultures of strain *Escherichia coli* SM10 λ_{pir} (pLOF/Km) and J105 (grown in Luria broth [LB] and LB20 [40], respectively, with appropriate antibiotics) were subcultured without antibi-

otics and grown to mid-log phase at 30°C with gentle shaking. A 200- μ l portion of the donor and a 100- μ l portion of the recipient strain were added to a 5-ml volume of a conjugation wash solution prepared by mixing 1 volume of marine minimal medium (40) and 1 volume of 10 mM MgSO₄. The cell suspension was filtered onto a membrane filter (GS Millipore; 0.22- μ m pore size; 5 cm²) and incubated on an LB15 plate (40) without antibiotics at 28°C for 4 h. The cells were thereafter resuspended in 1 ml of the conjugation wash solution and plated in serial dilutions on low-glucose X-Gal minimal-medium plates containing kanamycin and streptomycin at concentrations of 85 and 200 μ g $\cdot$ ml⁻¹, respectively. Several colonies that failed to become blue after prolonged incubation (i.e., 48 h) were detected; these were considered mutants potentially affected in a circuit with a positive regulatory role. To identify regulatory circuits essential for the development of a successful starvation-resistant phenotype, mutants with a pronounced defect in starvation survival were sought. The ability to survive carbon starvation was measured in CFU. One of the constitutively LacZ-negative double mutants (strain P10) exhibited a significant decrease in viability in comparison with the wild type (data not shown). Southern analysis of the genomic DNA extracted from strain P10 verified that the size of a 12-kb DNA *Eco*RI fragment containing the original mini-Mu(*lac*) transposon was unaltered in comparison with the parental strain, J105. It could therefore be concluded that the mini-Tn10 insertion had occurred in a locus distinct from the *csi-5::MudI(lac)* locus (data not shown). To verify the failure of strain P10 in expressing the *lacZ* gene of the *csi-5::MudI(lac)* locus upon carbon starvation, a single-cell β -galactosidase assay with high sensitivity was used (38). The result was consistent with that of the X-Gal plate assay and showed that no increase in β -galactosidase activity occurred when strain P10 was subjected to carbon starvation (4) (data not shown).

Identification of the gene interrupted by the secondary transposon in strain P10. The genomic DNA of strain P10 was digested with *Eco*RI, which does not cut within the sequence of the mini-Tn10(Km) transposon, and ligated into a pUC19 vector. After transformation of *E. coli* (XL-1 Blue) the resulting genomic library was plated on LB medium to select for kanamycin-resistant clones. Colony hybridization and restriction digestion analysis revealed that one such clone, designated pJO120, harbored a pUC19 plasmid with a 3.1-kb insert including the 1.9-kb mini-Tn10(Km) sequence. To sequence the DNA adjacent to the mini-Tn10(Km) insertional point, a sub-

* Corresponding author. Mailing address: School of Microbiology and Immunology, University of New South Wales, Sydney 2052, Australia. Phone: 61 2 3852102. Fax: 61 2 3136528. Electronic mail address: s.kjelleberg@unsw.edu.au.

† Present address: Centro de Investigaciones Biológicas, CSIC, 28006 Madrid, Spain.

TABLE 1. Strains, plasmids, and transposon elements used

	Description and/or relevant genotype	Source or reference
Strains		
<i>E. coli</i>		
SM10 λ_{pir}	Sm ^s , mobRP4, π -replicase (pir)	16
XL-1 Blue	Electrocompetent Epicurian	Stratagene
<i>Vibrio</i> sp.		
S14	Wild type, Sm ^s Km ^s Lac ⁻	19
S141	S14 spontaneously resistant to streptomycin	40
J105 ^a	S141 <i>csi-5::Mu dI(lac)</i>	40
P10	S141 <i>csi-5::Mu dI(lac) csrS::Tn10d(Km)</i>	This study
J109	S141 <i>csrS1 [csrS::Tn10d(Km)]</i>	This study
Plasmids		
pLOF/Km	Mini-Tn10(Km), Km ^r Ap ^r , <i>oriT</i> from mobRP4, R6K	16
pGP704	Ap ^r , <i>oriT</i> from mobRP4, R6K	25
pUC19	Ap ^r	Pharmacia
pJO120	pUC19 + a 3.1-kb <i>EcoRI</i> fragment from P10 genomic DNA	This study
pJO1201	pJO120 with a <i>HindIII</i> deletion	This study
pJO709	pGP704 with the 3.1-kb <i>EcoRI</i> insert from pJO120	This study
Transposons		
Tn10d(Km)	Mini-Tn10(Km), Km ^r , 1.9 kb	16
MudI(<i>lac</i>)	Mini-Mu (Tet ^r); originates from Mu dI1681; 17.1 kb	5

^a Previously designated S141 M5.

clone (pJO1201) in which one of the identical *IS10* ends of the transposon was deleted was constructed. This enabled the use of a DNA sequencing primer (5'-TTTACCAAAATCATT AGGGG-3') complementary to the remaining *IS10* sequence. The deduced amino acid sequence of the gene interrupted by the transposon contained 74% identity and, in view of the conserved amino acid substitutions, 85% similarity within the 119 codons sequenced to the *spoT* gene previously identified in *E. coli* (33) (Fig. 1). Furthermore, alignment of the obtained sequence to the *spoT* gene of *E. coli* showed that the presumed reading frame had been interrupted at a codon corresponding to approximately codon 360 in the *E. coli spoT* sequence (Fig. 1). The *E. coli spoT* gene encodes a protein that is 703 amino acids long, and therefore, the interruption at codon 360 suggests that 51% of the presumed *spoT* reading frame was uninterrupted. The insertionally destroyed allele was designated

```

Vibrio sp. S14 . . YTKMKNKEQRFHSIMDIYAFRVLVSDLDTCY 31
E. coli SIYCKMVLKEQRFHSIMDIYAFRVIVNDSDTCY 275

Vibrio sp. S14 RVLGQVHNLYKPRPSRMKDYYAIPKANGYQSLT 66
E. coli RVLGQMHSLYKPRPGRVKDYAIPKANGYQSLH 308

Vibrio sp. S14 TSLVGPHGVPEVQIRTEDMDQMAKVAHAHWT 97
E. coli TSMIGPHGVPEVQIRTEDMDQMAEMGVAHAHWA 339

Vibrio sp. S14 YKDGESSGTTAQVKAQPLDAKL>>miniTn10> 119
E. coli YKEHGETSTTAQIRAQRWMSLLELQSSAGSSF 370

```

FIG. 1. Amino acid sequence alignment of a region adjacent to the mini-Tn10(Km) insertion in strain P10 and the *E. coli spoT* gene as identified by Sarubbi et al. (32). Identical amino acids are indicated with dots, and similar amino acids are indicated with colons. Positions are given on the right. The estimated insertion point of the mini-Tn10(Km) transposon is indicated (>>). The amino acid sequence homology calculation was performed by using the algorithm of the GAP utility in the Genetics Computer Group software package (GCG; University of Wisconsin). Nucleotide sequence data have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence data libraries under the accession number U22374.

csrS1, and consequently, strain J109 herein is also referred to as the *csrS1* strain.

Transfer of the *csrS::Tn10d(Km)* allele into a *Vibrio* sp. strain S14 wild-type background. In order to study the biological effects of the mutation caused by the mini-Tn10 insertion in strain P10 without any interference from the *csi-5::MudI (lac)* fusion and other possible genotypic rearrangements, the *csrS::Tn10d(Km)* allele was transferred into the *Vibrio* sp. strain S14 wild type. The *EcoRI* insert was excised from pJO120 and ligated into pGP704 to construct pJO709, which was transformed into *E. coli* SM10 λ_{pir} . *E. coli* SM10 λ_{pir} carrying pJO709 was conjugated with *Vibrio* sp. strain S141, and transconjugants (recombinants) were isolated on LB20 plates by simultaneously selecting for kanamycin resistance and streptomycin resistance. To verify a double-crossover event, several transconjugants (recombinants) were subjected to colony hybridization in two replicas using two different probes, one specifically recognizing pGP704 DNA (full length) and the other specific for the Km gene of the mini-Tn10(Km) transposon (1.5-kb *NotI* fragment of pLOF/Km). One strain, designated J109, was found to be Km positive but negative for the pGP704 vector DNA, indicating that a double-crossover event had taken place. Southern analysis using the mini-Tn10(Km) probe, hybridized to two different restriction digests (*PvuII* and *EcoRI*) of the genomic DNAs from strains P10 and J109, verified that the mini-Tn10 insertion in strain J109 was identical to that of strain P10 (data not shown).

Cellular levels of ppGpp. The high similarity of the *csrS* gene of *Vibrio* sp. strain S14 to the *spoT* gene of *E. coli* is particularly intriguing. The activity of SpoT, i.e., the dephosphorylation of the signal molecule guanosine 3',5'-bispyrophosphate (ppGpp), is inversely related to, and/or complemented by, a ppGpp synthetase activity particularly in response to glucose starvation (15, 39). Assuming that the *csrS* gene is a homolog of the *spoT* gene in *E. coli*, and in view of failed attempts to create a *spoT* null mutant in the *E. coli* wild-type background (39), it is likely that the *csrS1* strain retains some SpoT activity. This argument is reasonable in light of findings that more than half of the

FIG. 2. Alterations in the ppGpp level (closed symbols) and growth curves (open symbols) for the wild-type *Vibrio* sp. strain S14 (circles) and strain J109 carrying the *csrS1* mutation (squares) before and after glucose exhaustion. Time of entry into stationary phase corresponds to 0 h. Cellular ppGpp was extracted and quantified by ion pair reverse-phase high-performance liquid chromatography as previously described (8). One OD₆₁₀ unit corresponds to cells in a 1-ml cell suspension of an optical density of 1, measured at 610 nm (LKB/Pharmacia Novaspec II). The wild-type ppGpp measurements have previously been published and are here plotted for comparative purposes.

putative *spoT* equivalent reading frame of the *csrS1* strain remained intact (see above). To determine whether the presumed SpoT activity of *Vibrio* sp. strain S14 was altered in the *csrS1* strain, the ppGpp content was monitored during log phase as well as during the initial phase of carbon starvation as previously described (8).

The basal level of ppGpp during logarithmic growth of the *csrS1* strain was found to be 42.0 ± 14 pmol per unit of optical density at 610 nm (OD₆₁₀) (Fig. 2). This is considerably higher than the basal level in the wild type, which previously has been measured to be 11.2 ± 2.2 pmol OD₆₁₀ unit⁻¹ (8). As in the wild type, a burst of ppGpp accumulation coincided with glucose exhaustion in the *csrS1* strain, but, while the amplitude of the ppGpp peak in the wild type previously has been found to be approximately 500 pmol OD₆₁₀ unit⁻¹ (8), it was found to be higher than 600 pmol OD₆₁₀ unit⁻¹ in the *csrS1* strain. These data are reminiscent of what previously was found in a run-out experiment with *E. coli* (6). However, the means of measuring and expressing the amount of ppGpp in relation to the OD of the sample was found to be incorrect, as the morphology and light scattering ability of the *csrS1* cells were altered in comparison with the wild-type cells (see Fig. 3). One OD₆₁₀ unit of a logarithmically growing *csrS1* culture corresponds to 1.17 ± 0.08 -fold more cells than 1 OD unit of the wild-type culture. Moreover, the mean cell volume of the growing *csrS1* cells was 1.41 ± 0.13 times greater than the mean volume of the corresponding wild-type cells. If ppGpp measurements are rectified by taking the above differences into account and estimating the intracellular concentrations of ppGpp, a somewhat different picture emerges. The ppGpp level detected in growing *csrS1* cells still proved to be higher than that in wild-type cells but only by a factor of approximately 2.1. However, the apparently higher amplitude of the ppGpp peak observed immediately after glucose exhaustion in the *csrS1* culture was now estimated to be approximately 1.3-fold lower compared with wild-type cells.

A regression analysis of the datum points obtained in the decline phase reveals that ppGpp levels out at a slower rate in the *csrS1* strain compared with the wild-type strain (Fig. 2). The *csrS1* cells undergo a relatively faster decrease in cell

volume, in relation to the change in the OD of the culture, than do the wild-type cells during the first hours of starvation. The actual rate of the ppGpp decline is therefore slower than indicated in Fig. 2. It is not certain whether this delayed decrease reflects a slower degradation rate, since it remains to be determined whether ppGpp decline following the initial burst is due to a change in synthesis or a change in degradation rate in wild-type cells. However, recent studies of several different *spoT* deletion mutants of *E. coli* suggest that the first half of the polypeptide is sufficient for dephosphorylation activity (ppGppase) whereas ppGpp synthetase activity requires some C-terminal amino acids for optimal activity (9). On the basis of this information it is likely that the *csrS1* strain is severely impaired in ppGpp synthetase activity and, to a minor extent, in the ppGppase activity of the presumed SpoT equivalent encoded by the *csrS* gene.

The generation times in marine minimal medium at 28°C were found to be 75 ± 3 and 65 ± 3 min for the *csrS1* strain and the wild-type strain, respectively. This observation is in accordance with the somewhat higher ppGpp level found during logarithmic growth of the *csrS1* strain, as it has previously been demonstrated for *E. coli* that the ppGpp level is inversely correlated to the mass of the translational apparatus and, hence, the potential growth rate at any given time (14, 20).

Morphological divergences. One of the characteristic starvation responses of *Vibrio* sp. strain S14 is the reductive division leading to starvation- and stress-resistant ultramicrocells (29). This response was studied by direct microscopic observations. Morphological differences between the wild type and the *csrS1* strain during growth and carbon starvation whereby the *csrS1* cells were always shorter and wider than the wild-type cells were observed (Fig. 3). The reason for these effects is not known, but, in view of the slow growth rate and assuming that the rate of DNA replication is not affected, the *csrS1* cell will not grow as long as the wild type before division and therefore probably appears more spherical. Alternatively, the spherical morphology might be due to a more specific mechanism related to the role of the *bolA* gene in *E. coli*. This gene is

time of starvation (hours)	wild type		J109	
	length $\pm$ sd width $\pm$ sd (μ m)	morphology	length $\pm$ sd width $\pm$ sd (μ m)	morphology
0 (log)	1.79 $\pm$ 0.20		1.53 $\pm$ 0.29	
	0.55 $\pm$ 0.06		0.73 $\pm$ 0.06	
1	1.53 $\pm$ 0.10		1.11 $\pm$ 0.21	
	0.45 $\pm$ 0.02		0.57 $\pm$ 0.08	
2.5	1.07 $\pm$ 0.16		0.72 $\pm$ 0.13	
	0.43 $\pm$ 0.01		0.45 $\pm$ 0.05	
24	0.94 $\pm$ 0.12		0.58 $\pm$ 0.18	
	0.41 $\pm$ 0.03		0.42 $\pm$ 0.05	

FIG. 3. Morphological observations during logarithmic growth (log) and carbon starvation (1, 2.5, and 24 h) for the wild-type *Vibrio* sp. strain S14 and strain J109 carrying the *csrS1* allele. The lengths and widths of the nondividing cells of each strain at different time points are represented graphically. The cells were killed by the addition of formaldehyde, to a final concentration of 4%, and cell morphology was observed, after heat fixation and staining with crystal violet, with a light microscope. Photographs were projected on a screen, allowing for accurate cell size measurements. At least 100 cells were measured in each sample, and data are expressed as means for two such samples $\pm$ standard errors.

FIG. 4. Changes in CFU with time of carbon starvation for the *Vibrio* sp. wild-type (wt) strain S14 (squares), the parental *lac* fusion strain J105 (triangles), and the *csrS1* strain J109 (diamonds). The values are expressed as percentages of the initial values obtained 40 h after the onset of starvation. At day 1 the OD of the starvation regimens was approximately 0.3 as measured at 420 nm (LKB/Pharmacia Novaspec II) and the CFU values were approximately 10^8 cells per ml. Duplicate experiments gave the same result.

required for the formation of coccoid morphology in stationary phase, and its expression is controlled from an inversely growth rate-dependent gearbox promoter (1, 2). The regulation of this promoter, *bolA1p*, is dependent on the sigma factor $\sigma^{80}/rpoS$ (23), whose synthesis is stimulated by ppGpp (10). Assuming that a gene equivalent to *bolA* exists in *Vibrio* sp. strain S14, the elevated ppGpp level of the growing *csrS1* cells may augment *bolA* expression and cause corresponding morphological alterations.

Viability. The *csrS1* strain exhibited a significant loss in viability during carbon starvation. The viability was reduced by a factor of at least 100, in comparison with the wild type, during the first 8 days after the onset of carbon starvation (Fig. 4). By monitoring the direct count (acridine orange), it was determined that the major loss of viability was not caused by cell lysis (data not shown). In this experiment, the starvation regimens were stored in glass flasks positioned on the laboratory bench top under the light of normal fluorescent tube lamps. However, when the same experiment was performed with the starvation regimens stored in complete darkness, no alteration in viability was observed for either the P10 strain or the *csrS1* strain (data not shown). Clearly, the inability to survive carbon starvation was due to difficulties in the development of starvation-induced protection against the stress imposed by visible light. It has been shown that visible light can generate photo-products that yield an oxidative stress with negative effects on gram-negative bacteria (3). Hydrogen peroxide (H_2O_2) is one of the photochemically generated products, and bacteria normally protect themselves by producing detoxifying enzymatic systems such as superoxide dismutase and catalases. We interpret these data as indicative of the failure of the *csrS1* strain to mobilize detoxifying enzymatic systems, DNA repair systems, or chaperone-related functions which have been shown to be induced by carbon starvation in bacteria (13, 17, 31).

Regulation of carbon starvation-specific responses. *csi-5* appears not to be induced in the *csrS1* background, which contains somewhat lower levels of ppGpp than the wild type upon glucose starvation. It might therefore be proposed that the up-regulation of the operon to which *csi-5* belongs is dependent on the ppGpp synthesis mediated by the presumed *Vibrio* *spoT* equivalent (*csrS*). However, this is probably not the case, as measurements of ppGpp accumulation upon glucose star-

vation of the *Vibrio* sp. strain S14 *relA* mutant show that ppGpp assumed to be synthesized by the equivalent of *E. coli* SpoT makes a very small contribution to the amplitude of the ppGpp peak seen directly after glucose exhaustion (8), when normally *csi-5* is induced. This conclusion is also supported by previous results demonstrating that the *csi-5* gene is not induced when the ppGpp level is increased as a result of amino acid starvation (40).

Although it has been found that many carbon starvation-induced proteins are under the control of RelA (and hence ppGpp) for full induction (28, 36), the data presented here demonstrating the failure of the *csrS1* strain to develop stress protection during starvation despite the burst and accumulation of ppGpp imply that signals in addition to ppGpp are needed. This reasoning is further supported by data obtained from stress experiments with the *Vibrio* sp. strain S14 *relA* mutant (8). This strain is severely impaired in ppGpp synthesis and yet shows few, if any, anomalies in terms of survival rate subsequent to exposure to various stress conditions (8). Furthermore, while amino acid starvation triggers ppGpp synthesis, it does not promote development of functions related to survival under conditions of carbon source and energy depletion (8). Since the *E. coli* SpoT protein may detect differences between carbon or energy source depletion and amino acid starvation (15, 39), it is postulated that the putative *spoT* equivalent, *csrS*, may mediate a carbon starvation-specific signal to play a pivotal role as a regulator of the adaptation to carbon starvation. It remains to be assessed whether the altered LacZ expression of strain P10 and/or the reduced survival phenotype of the *csrS1* strain is a direct consequence of the interruption of the *csrS* gene or if possible polar effects on the expression of nearby genes are of any significance. Vector systems available for complementation studies in the marine *Vibrio* sp. strain S14 should be developed to address these questions.

The inability of the *csrS1* strain to acquire the resistance necessary to withstand light exposure may also appear to be explained by an indirect metabolic effect. Overproduction of ppGpp in *E. coli* is known to affect expression of genes by a general decrease in translational efficiency (14). If the reduced declination rate of ppGpp after the onset of carbon starvation of the *csrS1* strain prolongs the decrease in the rate of protein synthesis, full induction of a crucial carbon starvation-induced protein may be disallowed and hence prevent the sequential starvation response. However, this does not explain why the *csi-5* operon is not induced in the *csrS1* background while the *csi-5::MudI(lac)* fusion is induced immediately after glucose exhaustion (40), when the amount of ppGpp in the *csrS1* strain is close to the wild-type levels.

In addition to the proposed specific role for the *csrS* gene product in mediating a successful adaptation to carbon starvation, it remains to be elucidated whether the enzymatic activities, related to ppGpp metabolism, of the gene product encoded by the *csrS* gene are also directly involved in regulating this response. Two-dimensional gel electrophoresis analysis of the carbon starvation response in the *csrS1* and *relA* mutant of *Vibrio* sp. strain S14 most likely would provide more detailed information on these questions.

We thank Victor de Lorenzo for providing strains and transposon vectors and Kathy Takayama for helpful discussions and critical reading of the manuscript.

This study was supported by grants from the Swedish Natural Science Research Council, the Swedish National Environmental Protection Agency (EU Biotechnology Programme), and the Australian Research Council.

REFERENCES

- Aldea, M., T. Garrido, C. Hernández-Chico, M. Vicente, and S. R. Kushner. 1989. Induction of a growth-phase-dependent promoter triggers transcription of *bolA*, an *Escherichia coli* morphogene. *EMBO J.* **8**:3923–3931.
- Aldea, M., C. Hernández-Chico, A. G. de la Champa, S. R. Kushner, and M. Vicente. 1988. Identification, cloning and expression of *bolA*, an *ftsZ*-dependent morphogene of *Escherichia coli*. *J. Bacteriol.* **170**:5169–5176.
- Arana, I., A. Muela, J. Iriberry, L. Egea, and I. Barcina. 1992. Role of hydrogen peroxide in loss of culturability mediated by visible light in *Escherichia coli* in a freshwater ecosystem. *Appl. Environ. Microbiol.* **58**:3903–3907.
- Barer, M. Personal communication.
- Belas, R., A. Mileham, M. Simon, and M. Silverman. 1984. Transposon mutagenesis of marine *Vibrio* spp. *J. Bacteriol.* **158**:890–896.
- Chaloner-Larson, G., and H. Yamazaki. 1978. Effects of the *spoT* and *relA* mutation on the synthesis and accumulation of ppGpp and RNA during glucose starvation. *Can. J. Biochem.* **56**:264–272.
- Errington, J. 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. *Microbiol. Rev.* **57**:1–33.
- Flärdh, K., T. Axberg, N. H. Albertson, and S. Kjelleberg. 1994. Stringent control during carbon starvation of marine *Vibrio* sp. strain S14: molecular cloning, nucleotide sequence, and deletion of the *relA* gene. *J. Bacteriol.* **176**:5949–5957.
- Gentry, D. R. Personal communication.
- Gentry, D. R., V. J. Hernandez, L. H. Nguyen, D. B. Jensen, and M. Cashel. 1993. Synthesis of the stationary phase sigma factor σ^s is positively regulated by ppGpp. *J. Bacteriol.* **175**:7982–7989.
- Givskov, M., L. Eberl, and S. Molin. 1994. Responses to nutrient starvation in *Pseudomonas putida* KT2442: two-dimensional electrophoretic analysis of starvation- and stress-induced proteins. *J. Bacteriol.* **176**:4816–4824.
- Givskov, M., L. Eberl, S. Møller, L. Kongsback Poulsen, and S. Molin. 1994. Responses to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content. *J. Bacteriol.* **176**:7–14.
- Hengge-Aronis, R. 1993. The role of *rpoS* in early stationary-phase gene regulation in *Escherichia coli* K12, p. 171–200. In S. Kjelleberg (ed.), *Starvation in bacteria*. Plenum Press, New York.
- Hernandez, V. J., and H. Bremer. 1990. Guanosine tetraphosphate (ppGpp) dependence of the growth rate control of *mnB* P1 promoter activity in *Escherichia coli*. *J. Biol. Chem.* **265**:11605–11614.
- Hernandez, V. J., and H. Bremer. 1991. *Escherichia coli* ppGpp synthetase II activity requires *spoT*. *J. Biol. Chem.* **266**:5991–5999.
- Herrero, M., V. de Lorenzo, and K. N. Timmis. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* **172**:6557–6567.
- Holmquist, L., Å. Jouper-Jaan, D. Weichert, D. R. Nelson, and S. Kjelleberg. 1993. The induction of stress proteins in three marine *Vibrio* during carbon starvation. *FEMS Microbiol. Ecol.* **12**:185–194.
- Holmquist, L., and S. Kjelleberg. 1993. The carbon starvation stimulon in the marine *Vibrio* sp. S14 (CCUG 15956) includes three periplasmic space protein responders. *J. Gen. Microbiol.* **39**:209–215.
- Humphrey, B., S. Kjelleberg, and K. C. Marshall. 1983. Responses of marine bacteria under starvation conditions at a solid-water interface. *Appl. Environ. Microbiol.* **45**:43–47.
- Kemp, P. F., S. Lee, and J. LaRoche. 1993. Estimating the growth rate of slowly growing marine bacteria from RNA content. *Appl. Environ. Microbiol.* **59**:2594–2601.
- Kjelleberg, S., T. Nyström, N. Albertson, and K. Flärdh. 1990. Nutrient limitation: global responses and prokaryotic development. *FEMS Microbiol. Ecol.* **74**:91–242.
- Lange, R., M. Barth, and R. Hengge-Aronis. 1993. Complex transcriptional control of the σ^s -dependent stationary-phase-induced and osmotically regulated *osmY* (*csi-5*) gene suggests novel roles for Lrp, cyclic AMP (cAMP) receptor protein-cAMP complex, and integration host factor in the stationary-phase response of *Escherichia coli*. *J. Bacteriol.* **175**:7910–7917.
- Lange, R., and R. Hengge-Aronis. 1991. Growth phase-regulated expression of *bolA* and morphology of stationary-phase *Escherichia coli* cells are controlled by the novel sigma factor σ^s . *J. Bacteriol.* **173**:4474–4481.
- Matin, A. 1990. Molecular analysis of the starvation stress in *Escherichia coli*. *FEMS Microbiol. Ecol.* **72**:185–196.
- Miller, M. L., and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170**:2575–2583.
- Neidhardt, F. C., J. L. Ingraham, and M. Schaechter. 1990. Physiology of the bacterial cell: a molecular approach, p. 351–388. Sinauer Associates, Inc., Sunderland, Mass.
- Nyström, T. 1993. Global systems approach to the physiology of the starved cell, p. 129–150. In S. Kjelleberg (ed.), *Starvation in bacteria*. Plenum Press, New York.
- Nyström, T. 1994. Role of guanosine tetraphosphate in gene expression and the survival of glucose or seryl-tRNA starved cells of *Escherichia coli* K12. *Mol. Gen. Genet.* **245**:355–362.
- Nyström, T., and S. Kjelleberg. 1989. Role of protein synthesis in the cell division and starvation induced resistance to autolysis of a marine *Vibrio* during the initial phase of starvation. *J. Gen. Microbiol.* **135**:1599–1606.
- Nyström, T., R. M. Olsson, and S. Kjelleberg. 1992. Survival, stress resistance, and alterations in protein expression in the marine *Vibrio* sp. strain S14 during starvation for different individual nutrients. *Appl. Environ. Microbiol.* **58**:55–65.
- Sak, B. D., A. Eisenstark, and D. Touati. 1989. Exonuclease III and the catalase hydroperoxidase II in *Escherichia coli* are both regulated by the *katF* gene product. *Proc. Natl. Acad. Sci. USA* **86**:3271–3275.
- Sarubbi, E., K. E. Rudd, and M. Cashel. 1988. Basal ppGpp level adjustment shown by new *spoT* mutants affect steady state growth rates and *mnA* ribosomal promoter regulation in *Escherichia coli*. *Mol. Gen. Genet.* **213**:214–222.
- Sarubbi, E., K. E. Rudd, H. Xiao, K. Ikehara, M. Kalman, and M. Cashel. 1989. Characterization of the *spoT* gene of *Escherichia coli*. *J. Biol. Chem.* **264**:15074–15082.
- Shimkets, L. J. 1990. Social and developmental biology of the myxobacteria. *Microbiol. Rev.* **54**:473–501.
- Siegele, D. A., M. Almirón, and R. Kolter. 1993. Approaches to the study of survival and death in stationary-phase *Escherichia coli*, p. 151–169. In S. Kjelleberg (ed.), *Starvation in bacteria*. Plenum Press, New York.
- Spector, M. P., and C. L. Cubitt. 1992. Starvation-inducible loci of *Salmonella typhimurium*: regulation and roles in starvation-survival. *Mol. Microbiol.* **6**:1467–1476.
- Spector, M. P., and J. W. Foster. 1993. Starvation-stress response (SSR) of *Salmonella typhimurium*: gene expression and survival during nutrient starvation, p. 201–224. In S. Kjelleberg (ed.), *Starvation in bacteria*. Plenum Press, New York.
- Walker, D. R., C. E. Nwoguh, and M. R. Barer. 1994. A microchamber system for the rapid cytochemical demonstration of beta galactosidase and other properties in pathogenic microbes. *Lett. Appl. Microbiol.* **18**:102–104.
- Xiao, H., M. Kalman, K. Ikehara, S. Zemel, G. Glaser, and M. Cashel. 1991. Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. *J. Biol. Chem.* **266**:5980–5990.
- Östling, J., A. Goodman, and S. Kjelleberg. 1991. Behaviour of IncP-1 plasmids and a miniMu transposon in a marine *Vibrio* sp.: isolation of starvation inducible *lac* operon fusions. *FEMS Microbiol. Ecol.* **86**:83–94.
- Östling, J., L. Holmquist, K. Flärdh, B. Svenblad, Å. Jouper-Jaan, and S. Kjelleberg. 1993. Starvation and recovery of *Vibrio*, p. 103–127. In S. Kjelleberg (ed.), *Starvation in bacteria*. Plenum Press, New York.