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Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*

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Summary

During carbon-starvation-induced entry into stationary phase, *Escherichia coli* cells exhibit a variety of physiological and morphological changes that ensure survival during periods of prolonged starvation. Induction of 30–50 proteins of mostly unknown function has been shown under these conditions. In an attempt to identify C-starvation-regulated genes we isolated and characterized chromosomal C-starvation-induced *csi*::*lacZ* fusions using the λp*lac*Mu system. One operon fusion (*csi*2::*lacZ*) has been studied in detail. *csi*2::*lacZ* was induced during transition from exponential to stationary phase and was negatively regulated by cAMP. It was mapped at 59 min on the *E. coli* chromosome and conferred a pleiotropic phenotype. As demonstrated by two-dimensional gel electrophoresis, cells carrying *csi*2::*lacZ* did not synthesize at least 16 proteins present in an isogenic *csi*2$^+$ strain. Cells containing *csi*2::*lacZ* or *csi*2::Tn*10* did not produce glycogen, did not develop thermotolerance and H_2O_2 resistance, and did not induce a stationary-phase-specific acidic phosphatase (AppA) as well as another *csi* fusion (*csi*5::*lacZ*). Moreover, they died off much more rapidly than wild-type cells during prolonged starvation. We conclude that *csi*2::*lacZ* defines a regulatory gene of central importance for stationary phase *E. coli* cells. These results and the cloning of the wild-type gene corresponding to *csi*2 demonstrated that the *csi*2 locus is allelic with the previously identified regulatory genes *katF* and *appR*. The *katF* sequence indicated that its gene product is a novel sigma factor supposed to regulate expression of catalase HPII and exonuclease III (Mulvey and Loewen, 1989). We suggest that this novel sigma subunit of RNA polymerase defined by *csi*2/*katF*/*appR* is a central early regulator of a large starvation/stationary phase regulon in *E. coli* and propose '*rpoS*' ('σ^S') as appropriate designations.

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Introduction

Escherichia coli can survive in such diverse environments as the mammalian gut or freshwater and must therefore possess powerful mechanisms to sense and react appropriately to dramatic changes in the availability of nutrients, osmolarity, temperature and other external factors. Among these, nutrient limitation appears to have the most global influence on cellular physiology and morphology.

Unlike, for instance, *Bacillus subtilis*, *E. coli* does not form endospores under starvation conditions and was therefore classified as a non-differentiating bacterium (Matin *et al.*, 1989). Nevertheless, morphological effects can be observed in starving, i.e. non-growing, stationary *E. coli* cells, which become smaller and sphere-shaped. Physiological adaptations, however, are most dramatic. During the transition into stationary phase glycogen is synthesized as a storage compound (Okita *et al.*, 1981) and the cells develop a remarkable resistance against heat shock and H_2O_2 (Jenkins *et al.*, 1988). Several stationary-phase-induced proteins have been reported, some of which are related to these phenotypes. Among these are catalase HPII (*katE*; Loewen *et al.*, 1985), exonuclease III (*xthA*; Sak *et al.*, 1989), acidic phosphatase (*appA*; Touati *et al.*, 1987), the microcins B17 and C7 (Genilloud *et al.*, 1989; Diaz-Guerra *et al.*, 1989), the morphogene BolA and FtsZ, a gene product involved in cell division (Aldea *et al.*, 1989). The corresponding genes, however, can only be a small subset of all stationary-phase-induced genes. On two-dimensional O'Farrell electrophoresis gels, 30–50 protein spots have been identified which were induced in *E. coli* and *S. typhimurium* cells entering stationary phase because of carbon limitation (Groat *et al.*, 1986; Spector *et al.*, 1986). Using multiple peptidase mutants, it was shown that protein degradation is required for survival of prolonged starvation (Reeve *et al.*, 1984), probably because synthesis of starvation proteins is dependent on endogenous amino acid resources created by proteolysis. Approximately two-thirds of these proteins are not found in an adenylate cyclase (*cya*)-defective mutant, indicating a control by cAMP and the catabolite activator protein (CAP, encoded by *crp*). Since *cya* and *crp* survive prolonged starvation periods equally well as wild-type strains, cAMP-dependent starvation proteins are not essential, at least under the starvation conditions tested (Schultz *et al.*, 1988). However, during glucose-starvation-induced entry

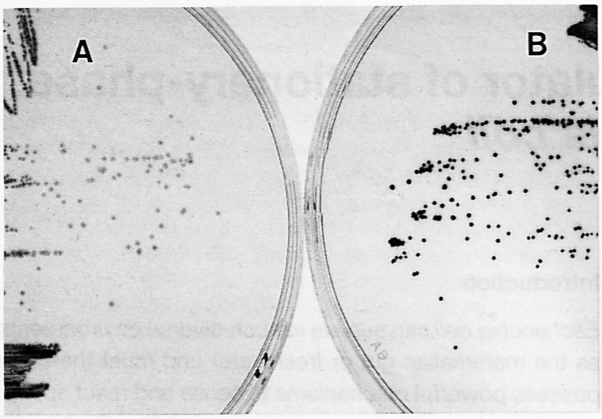

Fig. 1. Expression of *csi2*::*lacZ* on plates. MC4100 carrying *csi2*::*lacZ* was streaked on M9/XG plates containing 0.4% glucose (A) and 0.04% glucose ('starvation' plates; B).

into stationary phase, *E. coli* cells drastically increase their synthesis of cAMP, most of which is excreted into the medium (Buettner *et al.*, 1973). It is an unsolved paradox that cells appear to waste huge amounts of ATP to produce and excrete cAMP at a time when the energy source is becoming limited. The class of cAMP-independent starvation proteins includes the heat-shock proteins GroEL and DnaK and several other polypeptides that could also be induced by nitrogen and phosphate starvation (Schultz *et al.*, 1988).

The regulatory mechanisms responsible for the induction of starvation proteins can only be speculated about, since no general regulatory proteins have been identified so far. Guanosintetraphosphates have been implicated because a *relA*-independent increase in the intracellular ppGpp level correlates with the onset of starvation (Metzger *et al.*, 1989). Another potential factor in regulation is DNA topology. Plasmid DNA isolated from starving cells indicates a relaxation of DNA in stationary phase (Balke and Gralla, 1987). In the case of osmoregulated genes, it was demonstrated that changes in DNA supercoiling can have considerable effects on expression of global stimulons (Higgins *et al.*, 1988).

Using the λp*lac*Mu system (Bremer *et al.*, 1988) we isolated chromosomal *csi*::*lacZ* fusions. An adenylate-cyclase-defective mutant was used to identify cAMP-independent genes essential for survival during starvation. Several such *csi*::*lacZ* fusions have been characterized so far. This paper focuses on results obtained with one transcriptional fusion (*csi2*::*lacZ*) which allowed the identification of a central regulator of a large class of starvation proteins in *E. coli*.

Results

Isolation of csi::lacZ *fusions*

For the isolation of *csi*::*lacZ* fusions the randomly transposing λp*lac*Mu55 and λp*lac*Mu15 phages were used to obtain operon and protein fusions, respectively (Bremer *et al.*, 1988). *lacZ* fusions to *csi* genes were expected to synthesize higher levels of β-galactosidase on XG indicator plates with a low glucose concentration (0.02 or 0.04%; 'starvation' plates) than on plates with a high glucose content (0.4%). Since *lacZ* fusions to 'ordinary' catabolite-repressible operons (*mal*, *mgl*) were also induced under these conditions (our unpublished results), the isolation of *csi*::*lacZ* fusions was carried out in an adenylate-cyclase-deficient (Δ*cya*) strain in which neither catabolite-repressible operons nor non-essential cAMP/CAP-dependent starvation genes are expressed.

A Δ*cya* strain does not synthesize LamB, which is required as a receptor for λp*lac*Mu phages. We therefore introduced *zjb729*::Tn*10*, which is located in the intergenic region between *malK* and *lamB* and allows low-level constitutive expression of LamB under the control of the Tn*10* p_{out} promoter (Brass *et al.*, 1984).

Using this strain (RO1) as a receptor for λp*lac*Mu phages and the plate screen mentioned above, several chromosomal *csi*::*lacZ* fusions were isolated and characterized. All *csi*::*lacZ* fusions were P1-transduced into the wild-type strain MC4100 and into isogenic Δ*cya* strains (RO1, RH76). Preliminarily these fusions have been designated with numbers. The fusion *csi2*::*lacZ* characterized in this paper is a transcriptional fusion obtained with λp*lac*Mu55.

Kinetics of csi2::lacZ *expression*

A reduction in growth rate appeared to be the signal for induction of *csi2*::*lacZ*. This was demonstrated by the high β-galactosidase activity on starvation plates (0.04% glucose) where colonies exhibited a reduced growth rate limited by the diffusion of glucose (Fig. 1). In liquid minimal medium, however, fast growth stopped abruptly after exhaustion of glucose and only weak expression of *csi2*::*lacZ* was observed (Fig. 2A). Introduction of an adenylate cyclase mutation (Δ*cya*) reduced the growth rate of the *csi2*::*lacZ*-carrying strain (but not of a *csi*$^+$ strain) and resulted in increased expression of the fusion (Fig. 2B). In liquid rich medium, transition into stationary phase was accompanied by a gradual reduction of growth rate, during which a five-fold induction of *csi2*::*lacZ* was observed (Fig. 2C). During fast exponential growth no β-galactosidase was synthesized and pre-existing activity was diluted by cell division. In a similar experiment the early exponential phase was prolonged by repeated dilution and a basal expression level of 0.1 μmol min^{-1} mg^{-1} was determined (data not shown). Two-fold induction of *csi2*::*lacZ* could be obtained by a carbon 'downshift' from glucose to succinate, which also reduced the growth rate. When cells growing on minimal medium

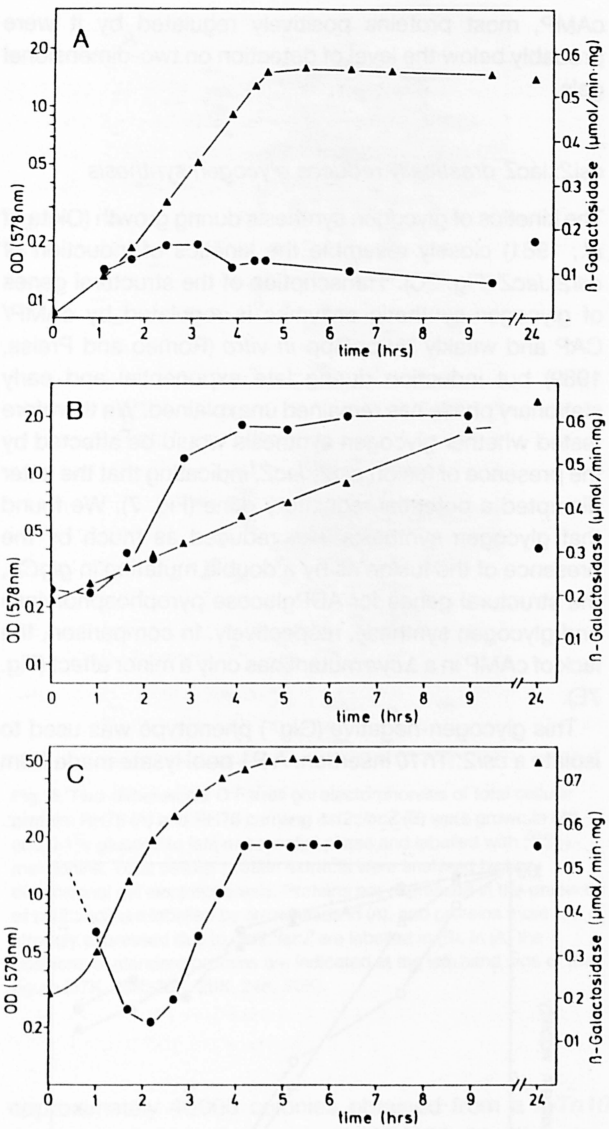

Fig. 2. Kinetics of induction of *csi2*::*lacZ*. Expression of *csi2*::*lacZ* was measured in MC4100 (*cya*$^+$) (A, C) and RO1 (Δ*cya*) (B). Cells were grown in M9 containing 0.1% glucose (A, B) or LB (C). OD_{578} was followed (triangles), and specific β-galactosidase activity was determined (circles).

with glucose were transferred to fresh medium without any carbon source, growth stopped immediately and no induction of *csi2*::*lacZ* was observed. Transfer into a phosphate-free medium (Groat *et al.*, 1986) resulted in induction of *csi2*::*lacZ* (to the fully induced level of 0.4–0.5 µmol min^{-1} mg^{-1}). In an adenylate-cyclase-deficient strain, however, phosphate starvation did not further increase the expression of the already induced *csi2*::*lacZ* fusion. Abrupt deprivation of nitrogen sources did not induce *csi2*::*lacZ* either in 'downshift' experiments or in cultures growing with a limiting concentration of ammonium chloride during (rather abrupt) entry into stationary phase. On the other hand, induced levels of β-galactosidase activity were observed in a culture growing slowly (with a doubling time of 140 min) with 2 mM glutamine as a nitrogen source or during gradual transition into stationary phase of a culture supplemented with a limiting concentration of yeast extract as a complex nitrogen source (data not shown).

csi2::lacZ *is negatively regulated by cAMP*

Surprisingly, we found higher expression and late exponential-phase induction of *csi2*::*lacZ* in a Δ*cya* strain (Fig. 2B). This suggested negative control by cAMP and we therefore tested the effect of cAMP directly. Strain MC4100 Δ*cya csi2*::*lacZ* was grown on minimal glucose medium and cAMP was added during mid-exponential phase. Expression of *csi2*::*lacZ* stopped immediately and pre-existing β-galactosidase was diluted during further growth (Fig. 3). Moreover, growth accelerated, indicating that at least in a Δ*cya* mutant, the gene disrupted by *csi2*::*lacZ* plays an important role during exponential growth in glucose minimal medium.

Chromosomal location of csi2::lacZ

Fusion *csi2*::*lacZ* was mapped on the *E. coli* chromosome using a collection of Tn*10* insertions distributed over the entire chromosome (Singer *et al.*, 1989). For approximate mapping, strains carrying Tn*10* insertions within 10-min intervals were pooled, whereas for fine mapping, single Tn*10* insertions were used. *csi2*::*lacZ* maps almost

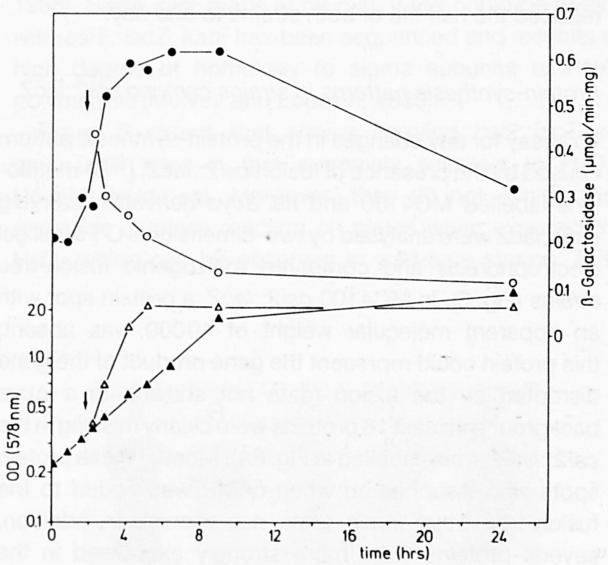

Fig. 3. *csi2*::*lacZ* is negatively regulated by cAMP. RO1 (Δ*cya*) carrying *csi2*::*lacZ* was grown on M9 containing 0.1% glucose, and OD_{578} (triangles) and specific β-galactosidase activity (circles) were determined. At the time indicated by the arrow the culture was divided and cAMP (5 mM) was added (open symbols).

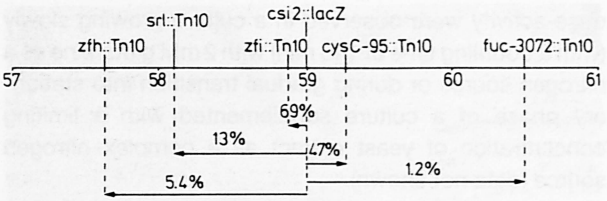

Fig. 4. Chromosomal location of *csi2*::*lacZ*. Co-transduction frequencies to Tn*10* insertions in marker genes of the 59-min region of the chromosome are shown. Arrowheads point to the selected markers in P1 transductions. Between 133 and 1645 transductants were screened for relevant markers in the various P1 transductions.

exactly at 59 min. Figure 4 shows co-transduction frequencies to surrounding gene markers and Tn*10* insertions. The gene order *srl–zfi*::Tn*10–csi2*::*lacZ–cysC* was confirmed by a three-factor cross in which a P1 lysate grown on a strain carrying *srl*::Tn*5* and *zfi*::Tn*10* (RO20) was transduced into strain MC4100 carrying *csi2*::*lacZ*, selecting for tetracycline resistance (Tc^R). Among Tc^R, Srl^- transductants, fusion-containing and fusion-free strains were obtained with equal frequency (3%), indicating a location of *csi2*::*lacZ* to the right of *zfi*::Tn*10*.

csi*2*::lacZ *reduces survival during prolonged starvation*

The loss of an essential *csi* gene due to disruption by *lacZ* can be expected to reduce the ability to survive prolonged starvation periods. Figure 5 demonstrates that this was the case for *csi2*::*lacZ*. Whereas cultures of a wild-type strain (MC4100) and an isogenic Δ*cya* derivative each exhibited a half-life of approximately six days, *csi2*::*lacZ* reduced the half-life of both strains to one day.

Protein-synthesis patterns in strains carrying csi*2*::lacZ

To assay for any changes in the protein-synthesis pattern caused by the presence of fusion *csi2*::*lacZ*, [^{35}S]-methionine-labelled MC4100 and its Δ*cya* derivative carrying *csi2*::*lacZ* were analysed by two-dimensional O'Farrell gel electrophoresis and compared to isogenic fusion-free strains (Fig. 6). In MC4100 *csi2*::*lacZ*, a protein spot with an apparent molecular weight of 40000 was absent; this protein could represent the gene product of the gene disrupted by the fusion (data not shown). In a Δ*cya* background at least 16 proteins were clearly missing in the *csi2*::*lacZ* strain (labelled in Fig. 6A). Most of these protein spots also disappeared when cAMP was added to the fusion-free Δ*cya* strain (data not shown). In addition, several proteins were more strongly expressed in the *csi2*::*lacZ* strain (labelled in Fig. 6B). We conclude that *csi2*::*lacZ* probably disrupts a regulatory gene that positively controls at least 16 other genes. Since in a *cya*$^+$ strain this regulatory gene is itself negatively controlled by cAMP, most proteins positively regulated by it were probably below the level of detection on two-dimensional gels.

csi*2*::lacZ *drastically reduces glycogen synthesis*

The kinetics of glycogen synthesis during growth (Okita *et al.*, 1981) closely resemble the kinetics of induction of *csi2*::*lacZ* (Fig. 2C). Transcription of the structural genes of glycogen-synthetic enzymes is regulated by cAMP/CAP and weakly by ppGpp *in vitro* (Romeo and Preiss, 1989) but induction during late exponential and early stationary phase has remained unexplained. We therefore tested whether glycogen synthesis would be affected by the presence of fusion *csi2*::*lacZ*, indicating that the latter disrupted a potential regulatory gene (Fig. 7). We found that glycogen synthesis was reduced as much by the presence of the fusion as by a double mutation in *glgCA*, the structural genes for ADPglucose pyrophosphorylase and glycogen synthase, respectively. In comparison, the lack of cAMP in a Δ*cya* mutant has only a minor effect (Fig. 7E).

This glycogen-negative (Glg^-) phenotype was used to isolate a *csi2*::Tn*10* insertion. A P1 pool lysate made from

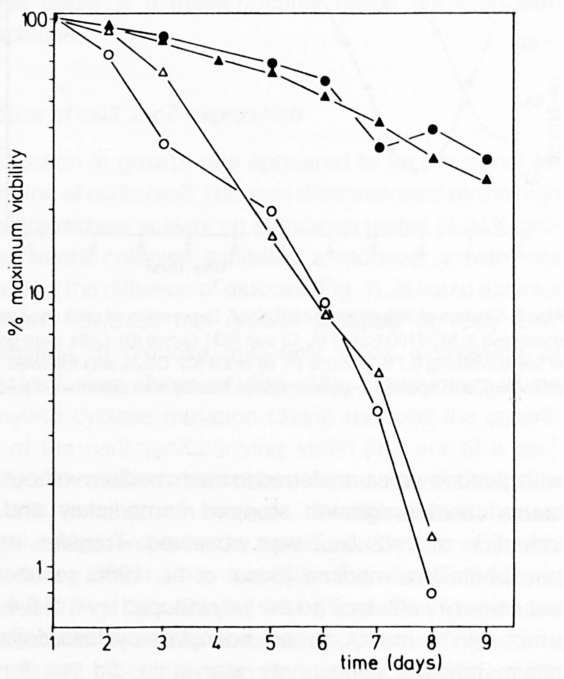

Fig. 5. Starvation survival of strains carrying *csi2*::*lacZ*. MC4100 (circles) and RO1 (Δ*cya*; triangles) carrying *csi2*::*lacZ* (open symbols) or no fusion (closed symbols) were grown on M9 and 0.1% glucose. After stationary phase was reached, incubation was continued for nine days under the same conditions. Viable cells were counted as colonies plated on LB-plates after appropriate dilution. One hundred percent viability corresponds to the number of viable cells counted one day after the culture had reached stationary phase.

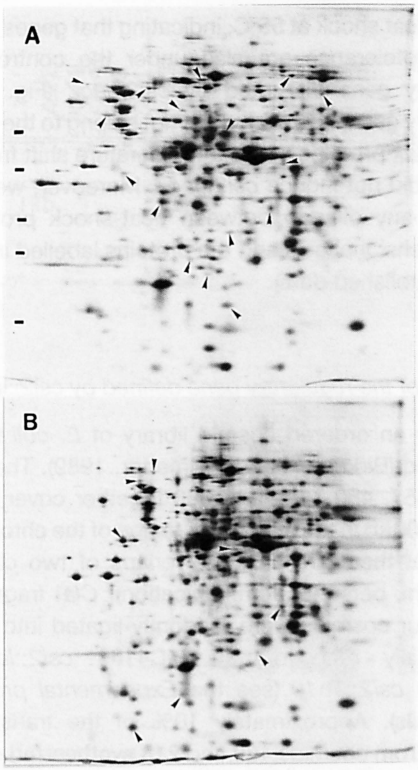

Fig. 6. Two-dimensional O'Farrell gel electorphoresis of total cellular protein. RH76 (A) and RH76 carrying *csi2*::*lacZ* (B) were grown in M9 and 0.1% glucose to late exponential phase and labelled with [^{35}S]-L-methionine. Total cellular protein extracts were analysed by two-dimensional gel electrophoresis. Proteins not expressed in the presence of *csi2*::*lacZ* are labelled by arrowheads in (A), and proteins more strongly expressed due to *csi2*::*lacZ* are labelled in (B). In (A) the positions of standard proteins are indicated at the left-hand side of the figure (67K, 45K, 36K, 29K, 24K, 20K).

approximately 40000 colonies obtained from a λ-Tn*10* 'hop' into the chromosome of MC4100 (M. Ehrmann, personal communication) was transduced into MC4100 carrying *csi2*::*lacZ*, and 40 white Tc^R colonies selected on XG/tetracycline indicator plates were purified. One of these 40 strains was Glg$^-$, indicating a *csi2*::Tn*10* insertion. This Tn*10* insertion could cross out *csi2*::*lacZ* with 100% efficiency in P1 transductions and exhibited the same co-transduction frequencies with nearby marker genes as *csi2*::*lacZ*. All phenotypes described in this paper for *csi2*::*lacZ* were also found for this Tn*10* insertion, which was therefore designated as *csi2*::Tn*10*. The other 39 Tn*10* insertions proved to be co-transducible with *csi2*::*lacZ*.

csi2::lacZ *inhibits the expression of a stationary-phase-specific acidic phosphatase (AppA)*

Fusion *csi2*::*lacZ* maps at the same chromosomal position as *appR*, a gene locus regulating *appA*-directed acidic phosphatase synthesis (Touati *et al.*, 1986). *appA* does not belong to the *pho* regulon, it is negatively regulated by cAMP, and it is induced when cells enter stationary phase (Touati *et al.*, 1987). We therefore tested whether *csi2*::*lacZ* and *appR* were allelic. Table 1 shows that stationary MC4100 *csi2*::*lacZ* cells exhibited a 14-fold reduction in acidic phosphatase activity relative to the wild-type strain. For spontaneous *appR* mutants, a four-fold reduction was reported (Touati *et al.*, 1986), indicating that these were not null mutants. This result indicated that *csi2*::*lacZ* and *appR* represent the same gene locus. This was further confirmed by the ability of a Δ*crp csi2*::*lacZ* strain but not of an isogenic fusion-free Δ*crp* strain to grow on succinate (data not shown), a phenotype described for *appR* Δ*crp* mutants (Touati *et al.*, 1986).

Table 1 also suggests that negative regulation of *appA* by cAMP is both indirect via regulation of *csi2*(*appR*) by cAMP and direct at the level of *appA* expression, since introduction of Δ*cya* or Δ*crp* into the *csi2*::*lacZ* strain resulted in a five-fold increase in AppA activity.

Strains carrying csi2::lacZ *or* csi2::*Tn*10 *do not develop* H_2O_2 *resistance*

Glucose-starved stationary wild-type cells exhibit a remarkable resistance against treatment with H_2O_2 (15–50mM; Jenkins *et al.*, 1988), which is dependent on catalase HPII and exonuclease III. These enzymes are encoded, respectively, by *katE* (Loewen *et al.*, 1985) and *xthA* (Sak *et al.*, 1989), both of which are positively controlled by *katF* (Sak *et al.*, 1989; Sammartano *et al.*, 1986). Since *katF* maps at 59 min, it too could be allelic with *csi2*::*lacZ*. *katF* has been sequenced and exhibits a high degree of homology to sigma subunits of RNA polymerase (Mulvey and Loewen, 1989).

Figure 8 shows that strains carrying *csi2*::*lacZ* or *csi2*::Tn*10* were in fact extremely sensitive to H_2O_2 (15mM) treatment. Moreover, they do not exhibit the explosive catalase reaction on plates when exposed to H_2O_2 which can be observed in wild-type strains. This

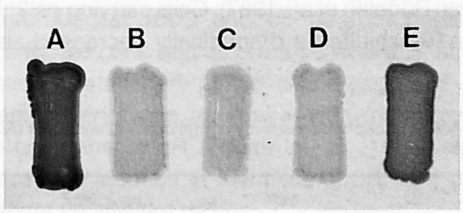

Fig. 7. Glycogen accumulation during growth on plates. The strains were grown overnight on glycogen test plates and stained with iodine vapors. A, MC4100; B, MC4100 *csi2*::*lacZ*; C, MC4100 *csi2*::Tn*10*; D, REI17 (*glgCA*); E, RH76 (Δ*cya*).

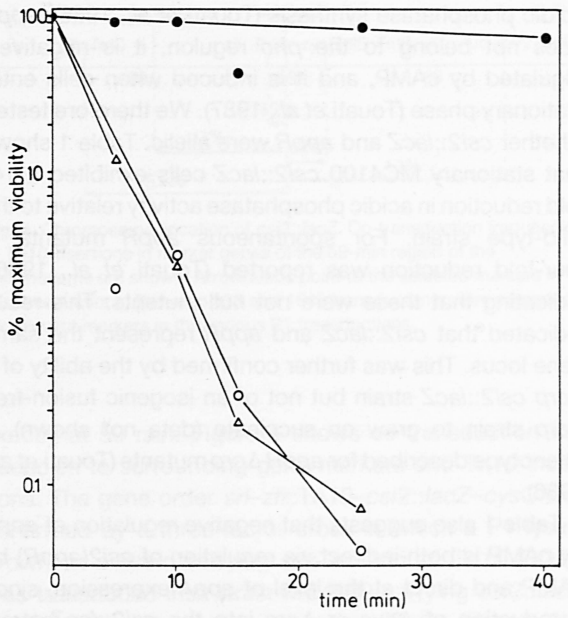

Fig. 8. Survival of H_2O_2 treatment. Stationary-phase cultures of MC4100 (closed circles), MC4100 *csi2*::*lacZ* (open circles) and MC4100 *csi2*::Tn*10* (open triangles) were exposed to 15 mM H_2O_2, and viable cell numbers were determined by diluting and plating aliquots onto LB-plates. One hundred percent viability corresponds to the viable cell number determined immediately before the addition of H_2O_2.

result and, finally, the comparison of the cloned *csi2* gene to the cloned *katF* unequivocally demonstrated that the two genes are identical (see below). Interestingly, addition of 60 μM H_2O_2 to growing cells did not have any influence on the level of expression of *csi2*::*lacZ* (data not shown) although under these conditions adaptive H_2O_2 resistance is observed (Jenkins *et al.*, 1988).

Strains carrying csi2::lacZ *or* csi2::*Tn*10 *are impaired in stationary-phase thermotolerance*

Upon entry into stationary phase, wild-type cells develop a pronounced tolerance against transient heat shocks at 55–57°C (Jenkins *et al.*, 1988). Although the heat-shock proteins GroEL and DnaK are induced in glucose-starved cells, induction of the classical *rpoH*-controlled heat-shock genes alone is not sufficient for this thermotolerance (van Bogelen *et al.*, 1987). Cells carrying *csi2*::*lacZ* or *csi2*::Tn*10* exhibited a dramatically decreased ability to survive heat shock at 55°C, indicating that genes involved in thermotolerance are also under the control of the regulatory gene disrupted by *csi2*::*lacZ* (Fig. 9). This regulatory gene, however, does not belong to the family of heat-shock proteins, since a temperature shift from 30°C to 42°C did not induce *csi2*::*lacZ*. Moreover, we did not observe any overlap between heat-shock proteins on two-dimensional gels and the proteins labelled in Fig. 6A (our unpublished data).

Table 1. Acidic phosphatase activity of stationary-phase cells (AppA; nmol min^{-1} mg^{-1}).

Strain	*csi*$^+$	*csi2*::*lacZ*
MC4100	38.2	2.7
MC4100 Δ*cya*	44.8	14.4
MC4100 Δ*crp*	67.7	17.7

Cloning of the regulatory gene defined by csi2::lacZ

Recently an ordered cosmid library of *E. coli* K12 was described (Birkenbihl and Vielmetter, 1989). The cosmid clones 557, 480, 218, and 333 together cover approximately 100 kb in the 58–60 min region of the chromosome such that there are always overlaps of two clones (R. Birkenbihl, personal communication). *Cla*I fragments of these four cosmids were randomly ligated into pBR322 and finally recovered in MC4100 *csi2*::*lacZ* and MC4100 *csi2*::Tn*10* (see the *Experimental procedures* for details). Approximately 10% of the transformants derived from cosmids 480 and 218 synthesized glycogen and exhibited catalase activity. Plasmids were prepared from six transformants derived from each cosmid. All 12 plasmids carried an identical 4.35 kb *Cla*I insert which had been present in both cosmids. A restriction map of this fragment is shown in Fig. 10A. An internal 2.4 kb *Kpn*I

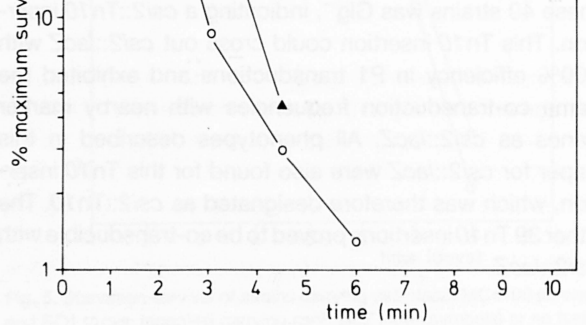

Fig. 9. Survival of heat shock at 55°C. Stationary-phase cells of MC4100 (closed circles), MC4100 *csi2*::*lacZ* (open circles) and MC4100 *csi2*::Tn*10* (triangles) were transferred to prewarmed tubes and viable cell numbers were determined by plating aliquots onto LB-plates. One hundred percent viability corresponds to the viable cell number determined immediately before heat shock.

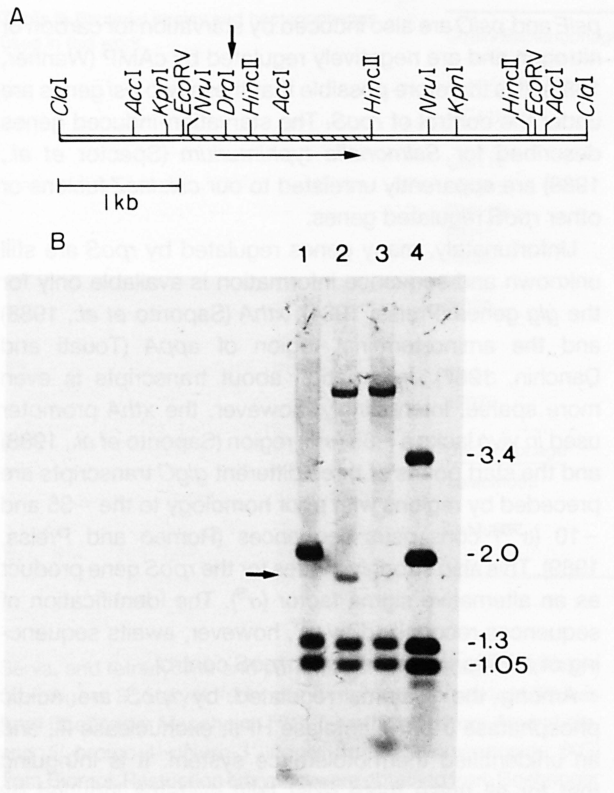

Fig. 10. Restriction map of the *csi2*::*lacZ* complementing *Cla*I fragment (A) and localization of *csi2*::*lacZ* within this fragment in the chromosome (B).

A. The 4.35 kb *Cla*I insert cloned into pBR322 (yielding pRH320) is shown. The cross-hatched fragment between the left *Nru*I site and the central *Hin*cII site corresponds to the region sequenced by Mulvey and Loewen (1989). The horizontal and the vertical arrows indicate the position of the open reading frame corresponding to *katF*/*csi2* and the position of the *csi2*::*lacZ* insertion in the corresponding region in the chromosome, respectively.

B. Chromosomal DNA of MC4100 (1), MC4100 *csi2*::*lacZ* (2), MC4100 *csi2*::Tn*10* (3) and pRH320 plasmid DNA was digested with *Cla*I and *Nru*I. Agarose gel electrophoresis and Southern transfer were followed by hybridization to the digoxigenine-labelled total *Cla*I insert of pRH320. Also a vector band (3.4 kb) is visible because of slight contamination of the probe with pBR322 vector sequences and can be used as a size standard. The arrow indicates a fragment (1.8 kb) from which the position of the fusion joint can be derived (see text).

fragment is in good agreement with the restriction map of a fragment carrying *katF* (Mulvey and Loewen, 1989). The internal 2.9 kb *Eco*RV and 2.0 kb *Nru*I fragments, both of which carry *katF*, were subcloned into pBR322, and complemented the glycogen- and catalase-negative phenotypes conferred by *csi2*::*lacZ* and *csi2*::Tn*10*. Southern hybridization experiments (Fig. 10B) with *Cla*I/*Nru*I-digested chromosomal DNA from wild-type and *csi2*::*lacZ* and *csi2*::Tn*10* insertion strains and the 4.35 kb *Cla*I fragment as a probe indicated that both insertions are within the 2.0 kb *Nru*I fragment. The first restriction site cut within λp*lac*Mu55 under these conditions is a *Cla*I site 1.4 kb downstream of the fusion point, and the last one is a *Cla*I site 3.45 kb upstream of the end of λp*lac*Mu (Bremer *et al.*, 1988). The 1.8 kb fragment labelled with an arrow in Fig. 10B, therefore, indicated that the fusion joint of *csi2*::*lacZ* was 0.4 kb to the right of the left *Nru*I restriction site, i.e. within the *katF* open reading frame. This demonstrated unequivocally that *csi2* and *katF* are allelic.

Discussion

In the present paper we describe the isolation and characterization of a starvation-inducible *lacZ* fusion with an extremely pleiotropic phenotype. Strains carrying *csi2*::*lacZ* or a Tn*10* insertion in the same gene were defective in a variety of stationary-phase-specific functions, among these glycogen synthesis, H_2O_2 resistance, thermotolerance, and activity of acidic phosphatase (AppA). They were seriously impaired in their ability to survive prolonged starvation periods and were deficient in synthesis of at least 15–20 proteins, as demonstrated by two-dimensional gel electrophoresis. These phenotypes point to a role as a central regulator of starvation functions for the gene disrupted by *csi2*::*lacZ*.

Fusion *csi2*::*lacZ* maps at the same location (59 min on the *E. coli* chromosome) as *katF*. This gene positively regulates catalase HPII (*katE*) and exonuclease III (*xthA*), which are required for H_2O_2 resistance (Sak *et al.*, 1989). Cloning of the gene from the 59-min region which complemented the *csi2*::*lacZ* phenotypes established its identity with *katF*. Also, the fusion joint of *csi2*::*lacZ* could be localized within the *katF* open reading frame. The *katF* sequence exhibits high homology to *rpoD*, which encodes the 'house-keeping' sigma subunit of RNA-polymerase (σ^{70}) in *E. coli*. It was suggested, therefore, that *katF* acted as a minor sigma factor in the control of an unknown protection or repair regulon to which *katE* and *xthA* belong (Mulvey and Loewen, 1989). In the light of our results it becomes clear that *csi2*/*katF* is a major regulatory gene for a large starvation regulon that comprises practically all stationary-phase-specific functions of *E. coli* described so far. In view of its sequence we consider it more than likely that the *csi2*/*katF* gene product acts as a sigma factor modifying the promoter-sequence recognition of RNA polymerase during transition into stationary phase. We therefore suggest '*rpoS*' and 'σ^S' as appropriate designations for the gene and its product, where 'S' stands for starvation or stationary phase. Since *csi2*::*lacZ* exhibits the same phenotypes as strains with mutations in the regulatory gene *appR* (Touati *et al.*, 1986) and their chromosomal location is identical, *appR*, too, probably represents an allele of *rpoS*.

The induction profile of the operon fusion *csi2*::*lacZ* indicated that *rpoS* is induced at the transcriptional level during transition from the exponential phase into the

stationary phase. Induction could be observed irrespective of whether the limiting compound was a source of carbon, of nitrogen or of phosphate. The inducing signal appears to be a reduction in growth rate, since various treatments with this effect, such as growth on plates with low glucose, carbon-source downshifts from glucose to succinate, or slow growth with glutamine as a nitrogen source, also resulted in induction. In various media, such as LB or minimal medium with a limiting concentration of complex nitrogen sources, transition into stationary phase is characterized by a gradual reduction in growth rate and results in induction of *csi*2::*lacZ*. Abrupt deprivation of carbon or nitrogen sources, which results in an immediate growth stop, did not induce *csi*2::*lacZ*. Interestingly, in similar phosphate-starvation experiments, increased expression of *csi*2::*lacZ* was observed. The kinetics of *csi*2::*lacZ* induction under various conditions show that exponentially growing cells maintain a low basal level of *rpoS* expression. Although we have not been able to detect any deficiency due to *csi*2::*lacZ* in exponentially growing wild-type cells, a three-fold increase in doubling time and a higher expression of *csi*2::*lacZ* in a Δ*cya* mutant could indicate that *rpoS* also plays a role during growth in an adenylate-cyclase-deficient mutant.

rpoS is negatively controlled by cAMP. The negative cAMP effect is partly reflected in the regulation of structural genes of the *rpoS* regulon. *appA* is repressed by cAMP, probably indirectly via the cAMP effect on *rpoS* expression as well as by a direct cAMP/CAP interaction with *appA* promoter sequences (Touati and Danchin, 1987). Also, negative regulation by cAMP might explain induction of *csi*2::*lacZ* by abrupt starvation for phosphate. Adenylate cyclase is stimulated by phosphate (Peterkofsky *et al.*, 1989). A sudden lack of phosphate may therefore result in a decrease in the intracellular level of cAMP, thus allowing better expression of *csi*2::*lacZ*.

For the glycogen-synthetic genes (*glgB*, *glgCA*), direct positive control by cAMP/CAP has been observed in *in vitro* transcription/translation assays (Romeo and Preiss, 1989). However, we observed only a minor reduction of glycogen synthesis in Δ*cya*, Δ*crp* or Δ*cya*Δ*crp* mutants, compared to a dramatic reduction caused by disruptions in *rpoS*. *rpoS* may be absolutely required for the expression of the *glg* genes, or for the expression of a factor required for activation of the *glg*-encoded enzymes. The same absolute requirement was found for H_2O_2 resistance, thermotolerance and for expression of another starvation-inducible operon fusion (*csi*5::*lacZ*) that maps around 99 min on the chromosome (our unpublished data).

A series of phosphate-starvation-inducible (*psi*) *lacZ* fusions was described by Wanner and McSharry (1982). Although none of our *csi*::*lacZ* fusions mapped at the chromosomal locations of any of the described *psi* genes, *psiE* and *psiO* are also induced by starvation for carbon or nitrogen and are negatively regulated by cAMP (Wanner, 1983). It is therefore possible that these two *psi* genes are under the control of *rpoS*. The starvation-induced genes described for *Salmonella typhimurium* (Spector *et al.*, 1988) are apparently unrelated to our *csi*::*lacZ* fusions or other *rpoS* regulated genes.

Unfortunately, many genes regulated by *rpoS* are still unknown and sequence information is available only for the *glg* genes (Preiss, 1984), *xthA* (Saporito *et al.*, 1988) and the amino-terminal region of *appA* (Touati and Danchin, 1987). Information about transcripts is even more sparse. Interestingly, however, the *xthA* promoter used *in vivo* lacks a −35 (σ^{70}) region (Saporito *et al.*, 1988) and the start points of three different *glgC* transcripts are preceded by regions with poor homology to the −35 and −10 (σ^{70}) consensus sequences (Romeo and Preiss, 1989). This also strongly argues for the *rpoS* gene product as an alternative sigma factor (σ^S). The identification of sequences recognized by σ^S, however, awaits sequencing of additional genes under *rpoS* control.

Among the systems regulated by *rpoS* are acidic phosphatase (AppA), catalase HPII, exonuclease III, and an unidentified thermotolerance system. It is intriguing that for all these there exist twin systems induced by specific stresses. These include alkaline phosphatase which is controlled by *phoR*/*phoB* (Wanner, 1987), the *oxyR*-regulated catalase HPI (Christman *et al.*, 1985), various DNA-repair systems that belong to the *lexA*/*recA*-dependent SOS regulon (Walker, 1987), and the heat-shock system, which is under the control of another alternative sigma factor, *rpoH* (Neidhardt and van Bogelen, 1987). Whereas these systems are induced in reaction to phosphate limitation, oxidative stress, DNA damage and temperature shifts, respectively, the *rpoS*-dependent systems with similar functions are not induced by these specific stimuli. It appears to have been more 'economic' to evolve alternate systems for all these functions, rather than subjecting single systems to highly complex multiple controls. In this respect, the *rpoS*-regulated functions belong to a 'superglobal' regulatory programme comparable in its complexity and physiological significance to the sporulation programme of *B. subtilis*. It is possible that in this programme *rpoS* is the first regulatory gene followed by other unidentified regulators.

Experimental procedures

Media and chemicals

Media were purchased from Difco and prepared according to Miller (1972). Concentrations of carbon sources and supplements are indicated in the various experiments. Kanamycin and ortho-nitrophenyl-galactopyranoside (ONPG) were purchased from

Table 2. Bacterial strains and bacteriophages used in this work.

Strain/Bacteriophage	Relevant genotype	Source
E. coli K12		
MC4100	F⁻, (*argF–lac*)*U169*, *araD139*, *rpsL150*, *ptsF25*, *flbB5301*, *rbsR*, *deoC*, *relA1*	Silhavy *et al.* (1984)
GM114	MC4100, *zfi-551*::Tn*10*	G. May *et al.* (1986)
GM115	MC4100, *zfh-552*::Tn*10*	G. May *et al.* (1986)
GM119	MC4100, *srl*::Tn*10*	G. May *et al.* (1986)
REI17	MC4100, *glgAC*, *malK*::*lacZ*, *mall*, *ugp*::Tn*10*	J. Reidl
RH76	MC4100, Δ*cya851*	This study
RO1	RH76, *zjb-729*::Tn*10*	This study
RO20	F⁻, (*argF–lac*)*U169*, *araD139*, *rpsL150*, (*glpA–glpT*)*593*, *gyrA*, Δ*phoA8*, *recA*, *srl*::Tn*5*, *zfi-551*::Tn*10*	This study
12173	*cysC-95*::Tn*10*	Singer *et al.* (1989)
12079	*fuc-3072*::Tn*10*	Singer *et al.* (1989)
Bacteriophage		
λp*lac*Mu55	Mucts62, *ner*⁺, *A'*am1093, *'uvrD'*, Mu*S''trp'*, *lacZ*⁺, *lacY*⁺, *lacA'*, *imm*λ	Bremer *et al.* (1988)
λpMu507	Mucts62, Mu*A*⁺*B*⁺, λ*c*ts857, *S*am7	Magazin *et al.* (1977)

Serva, and tetracycline and para-nitrophenyl-phosphate (PNPP) from Sigma. 3',5'-cyclo-adenosine-monophosphate (cAMP) was from Boehringer Mannheim, [^{35}S]-L-methionine from Amersham, and 5'-bromo-4'-chloro-3'-indolyl-β-D-galactopyranoside (XG) from Biomol. Restriction enzymes were obtained from Boehringer Mannheim, BRL (*Hin*cII) or New England Biolabs (*Acc*I). T4-DNA ligase was from Pharmacia. Chemicals for two-dimensional gel electrophoresis were purchased from BioRad. All other chemicals were obtained from Merck.

Bacterial strains, phages and growth conditions

Bacteria were grown aerobically at 37°C, and growth was monitored by measuring optical density at 578nm (OD_{578}). Bacterial strains and phages used in this study are listed in Table 2.

Genetic techniques and DNA manipulations

For P1 transduction, standard techniques were used (Miller, 1972). Chromosomal *lacZ* fusions were isolated by co-infection with λpMu507 as a helper phage and the fusion phages λp*lac*-Mu55 or λp*lac*Mu15 and selection for kanamycin-resistant strains (Bremer *et al.*, 1988). For DNA manipulations, procedures described by Silhavy *et al.* (1984) and Sambrook *et al.* (1989) were followed.

For cloning of the wild-type gene from the 59-min region that complemented *csi*2::*lacZ* phenotypes, four cosmid clones (nos 557, 480, 218, and 333; R. Birkenbihl, personal communication) carrying DNA from this region were digested with *Cla*I, randomly ligated into pBR322, and transformed into a highly transformable strain. Approximately 1000 transformants derived from each cosmid were pooled, and the plasmids were prepared and transformed into MC4100(*csi*2::*lacZ*) and MC4100(*csi*2::Tn*10*). Replicas of the transformation plates were assayed for glycogen (Glg) accumulation and catalase (Cat) activity (see below). Glg⁺ Cat⁺ transformants were purified and used for plasmid preparation.

Chromosomal DNA was isolated using the procedure of Silhavy *et al.* (1984). For Southern transfer, labelling of probes, and hybridization, protocols provided by Sambrook *et al.* (1989) and Boehringer Mannheim (non-radioactive DNA labelling and detection kit) were followed.

Survival assays

Long-time starvation survival was assayed by growing cells in 50ml of M9 medium containing 0.1% glucose. During growth and then for up to 10d samples were withdrawn for determination of OD_{578} and viable cell numbers on LB-plates after appropriate dilution in 0.9% NaCl.

For the heat-shock survival assay, cells were grown overnight in LB. Stationary cells were washed and diluted in 0.9% NaCl to a density of about 5000 cells ml^{-1}. One-millilitre samples were put into prewarmed glass tubes (55°C) and at the times indicated 0.1ml portions were plated directly onto LB-plates to determine viable cell numbers.

For H_2O_2 treatment, cells were grown overnight in LB, washed and resuspended in 0.9% NaCl to an OD_{578} of 1.0. H_2O_2 was added to a final concentration of 15mM. At the times indicated, 0.1-ml samples were withdrawn, diluted immediately in 0.9% NaCl and plated onto LB-plates.

Plate assays

β-galactosidase activity was assayed qualitatively on various plates containing 50 μg ml^{-1} XG.

Accumulation of glycogen was tested by growing cells as single colonies or in patches on plates containing 1% glucose (Latil-Damotte and Lares, 1977). Instead of overlaying the plates with an iodine salt solution, they were stained with iodine vapours by exposure to crystalline iodine for 2–5 min. Glycogen-accumulating strains stain dark brown; staining is reversible, can be repeated several times, and does not kill the cells.

Catalase activity was tested qualitatively by applying small drops of H_2O_2 (30%) on to colonies or patches grown overnight on LB plates.

Enzyme assays

Quantitative β-galactosidase assays were performed as described by Miller (1972). Acidic phosphatase (AppA) was assayed in stationary-phase overnight cultures exactly as described by Dassa *et al.* (1982).

Two-dimensional O'Farrell gel electrophoresis

This was performed according to the method of O'Farrell *et al.* (1977). Cells were grown in M9 medium containing 0.1% glucose to late exponential phase. The OD_{578} of 1-ml samples was adjusted to 0.15 with the same medium. Ten to fifteen micro-Curies of [^{35}S]-L-methionine (>1000 Ci mmol^{-1}) was used to label a 1-ml sample for 2 min, followed by a 1-min chase with 0.2 mM unlabelled methionine. Samples were precipitated with trichloroacetic acid (10%), washed with acetone and resuspended in first-dimensional sample buffer. Two-dimensional gel electrophoresis was carried out in a BioRad mini-Protean device according to the directions given by the manufacturer. Separation in the first dimension was by non-equilibrium pH gradient electrophoresis, where the pH gradient ranged from approximately pH 4.8 to pH 7 (left and right side, respectively, in Fig. 6). Running conditions were as follows: 10 min of pre-electrophoresis at 500 V without a buffer change, 10 min of electrophoresis at 500 V, followed by 4 h 50 min at 750 V. In the second dimension, sodium dodecyl sulphate/polyacrylamide (12%) gels were run for 100 min at 135 V. Gels were stained with Coomassie Brilliant Blue and dried. Fuji X-ray films were used for autoradiography.

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