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surA, an *Escherichia coli* Gene Essential for Survival in Stationary Phase

ANTONIO TORMO,† MARTA ALMIRÓN, AND ROBERTO KOLTER*

Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115

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Mutations in genes not required for exponential growth but essential for survival in stationary phase were isolated in an effort to understand the ability of wild-type *Escherichia coli* cells to remain viable during prolonged periods of nutritional deprivation. The phenotype of these mutations is referred to as Sur$^-$ (survival) and the genes are designated *sur*. The detailed analysis of one of these mutations is presented here. The mutation (*surA1*) caused by insertion of a mini-Tn*10* element defined a new gene located near 1 min on the *E. coli* chromosome. It was located directly upstream of *pdxA* and formed part of a complex operon. Evidence is presented supporting the interpretation that cells harboring the *surA1* mutation die during stationary phase while similar insertion mutations in other genes of the operon do not lead to a Sur$^-$ phenotype. Strains harboring *surA1* had a normal doubling time in both rich and minimal medium, but cultures lost viability after several days in stationary phase. Analysis of revertants and suppressors of *surA1*, which arose after prolonged incubation in stationary phase, indicates that DNA rearrangements (excisions and duplications) occurred in cultures of this strain even when the viable-cell counts were below 10^2 cells per ml. Cells containing suppressing mutations then grew in the same culture to 10^8 cells per ml, taking over the population. The implications of these observations to our understanding of stationary-phase mutagenesis are discussed.

Bacterial cells are capable of extremely rapid metabolism, a fact that is displayed in doubling times as short as 15 min when conditions are favorable for growth. However, bacteria have also evolved diverse mechanisms to maintain the living state for prolonged periods under conditions which are not propitious for exponential growth. Aside from the metabolically dormant states achieved by sporulating microorganisms (25), it is clear that metabolically active yet nondividing states are maintained by numerous bacteria (35). Indeed, in their natural environment bacteria spend only a small fraction of their existence in exponential growth due to the limited availability of nutrients. The vast majority of their existence must, by necessity, be in a nongrowing state. Yet most of our understanding of bacterial physiology has been obtained from cells undergoing exponential growth.

Because of their obvious relevance to understanding the living state, the processes of cellular aging and death have been of great interest in microbiology. Much of the early work of bacterial physiology addressed questions regarding cell death and survival strategies, but it became apparent that even general questions such as distinguishing live cells from dead ones encountered substantial complications (35). With the advent of molecular biology and the ability to study regulatory processes in great detail, the major emphasis in recent years has been to study regulation in growing or transiently starved bacterial cells. With few exceptions, such as the studies of differentiation in sporulating bacteria (25), efforts to understand bacterial physiology have been focused on cells in balanced growth. In the case of the relatively well understood bacterium *Escherichia coli*, which does not obviously undergo cellular differentiation upon nutrient deprivation, the gap in our understanding of stationary phase versus exponential phase is particularly evident. Yet a number of reports suggesting the unusual appearance of mutations during stationary phase in *E. coli* (9, 18, 36, 38) point to a need to better understand this phase of cellular physiology.

Fortunately, there has been a recent resurgence of interest in studying stationary phase in *E. coli* with markedly more "molecular" approaches (27). The global analysis of proteins synthesized under a variety of different starvation conditions revealed a subset of proteins whose synthesis increased regardless of nutritional deprivation (17). While the identification and determination of the specific role of these proteins are not yet complete, the growth phase-dependent synthesis of several substances has been studied. Among these are glycogen (31), polyphosphates (32), acid phosphatase (11), catalase (24), and microcins B17 and C7 (14, 19). For some of these, it has been demonstrated that the increase in synthesis is due, at least in part, to specific activation of transcription at the onset of stationary phase. The microcin B17 promoter was perhaps the first growth phase-dependent promoter sequenced (10). Its sequence did not match the consensus of a typical σ^{70} promoter, suggesting that perhaps there are one or more "stationary phase" σ factors. Similar promoters have been found in several other *E. coli* genes, among them the cell division genes *bolA*, *ftsAZ*, and *ftsQ*, indicating the existence of at least one growth phase-dependent regulon (1). A putative stationary-phase σ factor (the product of the *katF* gene) has been shown to be involved in the expression of the catalase structural gene, *katE* (30). In the case of acid phosphatase and microcin C7, the transcription of their structural genes is stimulated by the product of the *appR* gene (14, 41). In addition, another gene (*appY*) has been identified which, when cloned in high copy number, also affects the growth phase-dependent expression of the acid phosphatase gene (5). Finally, the product of the *osmZ* gene has also been implicated in growth phase-dependent regulation (20).

While these studies of transcriptional regulation in station-

* Corresponding author.
† Present address: Departamento de Bioquímica, Universidad Complutense de Madrid, Madrid, Spain.

ary phase have served to define some of the processes that take place in nongrowing *E. coli* cells, they have not directly addressed the question of survival during prolonged periods of starvation. Under certain conditions of starvation, some strains of *E. coli* have been shown to enter an exponential death phase, and it has been argued that the death of the cell is due to the degradation of the protein-synthetic machinery (13). However, under most conditions, a typical laboratory "wild type" strain of *E. coli* can display a remarkable ability to remain viable.

It has been reported that upon treatment of a strain with chloramphenicol during the first hours of stationary phase, there is a more rapid loss of viability (33). This was interpreted as an indication that the proteins synthesized during the early part of stationary phase were in some way involved in the generation of a more resistant form of the bacterium. Consistent with this interpretation was the finding that some strains harboring Mu d*lac* insertions whose expression is increased during stationary phase can also display a decrease in viability upon prolonged incubation in stationary phase (17, 40). While the loss of viability after treatment with chloramphenicol or in the fusions is not complete, the results suggest an important role for protein synthesis during stationary phase for long-term cellular survival.

To help us address the issue of long-term cellular survival directly, we embarked on the identification of genes that are not essential during exponential growth but whose products are required in order to survive in stationary phase. We found several such genes, and in this article we report the isolation and characterization of strains mutated in one such gene.

MATERIALS AND METHODS

Bacterial media, culture assays, strains, and plasmids. Liquid and solid LB and M63-glucose media were prepared as described by Miller (28). When pyridoxine was used as a supplement, it was added at 1 μg/ml. The following concentrations of antibiotics were used: kanamycin, 50 μg/ml; chloramphenicol, 200 μg/ml; ampicillin, 150 μg/ml. Cultures to be incubated for prolonged periods consisted of 3 ml of liquid medium in glass test tubes (18 by 200 mm). Cultures were kept aerated by rotation in a New Brunswick gyrotory wheel at 37°C. Viable-cell counts were determined by spot-plating dilutions from these cultures onto appropriate solid medium. Experiments were repeated at least five times, and when results showed fluctuation, this is indicated in the Results section. Acridine orange (Difco Laboratories) staining of cultures was performed as described before (23), and cells were observed in a Zeiss fluorescence microscope. Renograffin 76 (Squibb Diagnostics) was used to make preformed step density gradients (1.125 to 1.225 g/ml). Samples were layered on top and centrifuged for 60 min at 75,000 rpm in a TLA-100 rotor in a TL-100 ultracentrifuge (Beckman). Bands were collected with a syringe and needle, assayed for viable-cell counts, and stained with acridine orange.

All bacterial strains used were *E. coli* K-12 derivatives. ZK126 is W3110 *tna-2* Δ*lacU169* and comes from our laboratory stocks (10); JC7623 is *arg ara his leu pro recB21 recC22 sbcB15 thr* (43); and NU811 is W3110 *pdxA*::Km^r (*Eco*RV) (34). Both JC7623 and NU811 were kindly provided by M. Winkler. We used strain 71-18 to propagate recombinant M13 phages (21). The phage used to deliver the mini-Tn*10* Km^r element into the chromosome was λ1105 (42). Random hops of this element were generated by the method described before (42). The cloning vectors pUC19 and M13mp19 have been described (21). The Kohara miniset of λ phages containing the ordered library of the W3110 chromosome (22) was kindly provided by Ken Rudd.

DNA analysis procedures. Restriction enzymes, T4 DNA ligase, and mung bean nuclease were purchased from New England BioLabs and used in the buffers recommended by the supplier. Sequencing was performed by the chain termination method (37) with Sequenase kits from U.S. Biochemical Corp. Southern hybridizations were performed as described before (39). Chromosomal and plasmid DNA preparation, nick translation, and plaque lifts onto nylon membranes were performed by the methods of Maniatis et al. (26).

Construction of insertion mutations in specific genes. In every case, the psurA$^+$ plasmid, containing the wild-type *Pst*I fragment which carries part of *surA* and *pdxA*, *ksgA*, *apaG*, and *apaH*, was linearized with a restriction enzyme with a unique recognition site. For each gene, the enzyme used was: *surA*, *Bam*HI; *ksgA*, *Pfl*MI; *apaG*, *Cla*I. A *Bam*HI fragment of 1,726 base pairs containing the kanamycin resistance gene and present in mini-Tn*10* (42) was purified and is referred to here as the kanamycin resistance cassette. The *Bam*HI ends were left intact for ligation into the *Bam*HI site in *surA*. For ligation into the *Pfl*MI and *Cla*I sites, both the linearized plasmid and the cassette were treated with mung bean nuclease prior to ligation to generate blunt ends. After ligation, the DNA was used to transform strain 71-18. For every construct, the plasmid with the cassette inserted so that transcription of the kanamycin resistance gene was in the same direction as transcription of the operon was selected. These plasmids were subsequently linearized with *Eco*RI (which cuts only the plasmid vector) and used to transform the *recBC sbcB* strain JC7623 to obtain recombinants in which the plasmid had integrated into the chromosome as described before (43). P1 *vir* was grown on these strains, and the kanamycin resistance marker was moved into ZK126 by transduction as described before (28). The expected physical map of the chromosomal DNA surrounding the site of cassette insertion was verified by Southern filter hybridization.

RESULTS

Long-term viability of the "wild type" ZK126. At the outset of these studies, it was necessary to choose a particular strain of *E. coli* which would be suitable for the analysis of stationary-phase survival. Because of the relative absence of mutagenic treatments in its laboratory history (6), its ability to survive prolonged incubations in the absence of growth (see below), and the knowledge of the complete restriction site map of its chromosome, we chose a derivative of W3110, designated ZK126, which carries the Δ*lacU169* deletion (10). This strain's ability to form colonies as a function of time when incubated at 37°C with aeration is shown in Fig. 1. Figure 1A represents growth in LB medium, while Fig. 1B represents growth in M63 minimal salts plus glucose, where glucose was limiting for growth.

In LB this strain reaches viable-cell counts in the vicinity of 10^{10} CFU/ml upon the cessation of exponential growth. For the first 4 days of incubation, these counts dropped exponentially to about 5×10^8 CFU/ml. However, once the viable counts reached about 10^8 CFU/ml, the death phase stopped and viable-cell counts remained virtually unchanged for many days. While Fig. 1A displays only the first 9 days of a typical culture, we monitored cultures for much longer

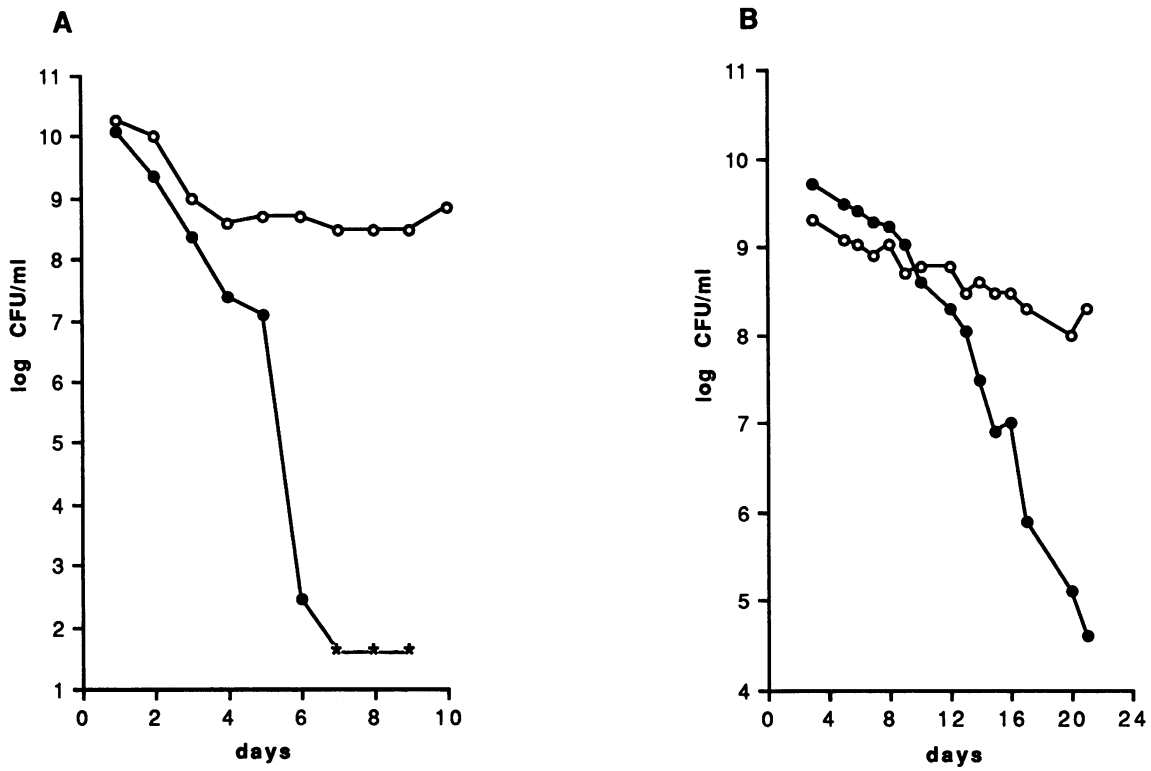

FIG. 1. Long-term viability of ZK126 (○) and ZK126 *surA1* (●). Viable-cell counts were determined by plating serial dilutions in plates of the same medium composition as used for the incubation. (A) Incubation in LB (ZK126) or LB plus kanamycin (ZK126 *surA1*). (B) Incubation in M63-glucose-pyridoxine minimal medium (kanamycin included for ZK126 *surA1*). The stars at $<10^2$ CFU/ml indicate that no colonies grew in the lowest dilution plated (10 μl directly from the culture).

periods of continued incubation. After 1 month there were still about 10^8 CFU/ml, and even after 1 year most cultures tested had titers of between 10^6 and 10^7 CFU/ml.

After growth in minimal M63 salts plus glucose (Fig. 1B), ZK126 did not undergo the early drop in viable-cell counts characteristic of growth in LB. Viable counts remained at about 10^9 CFU/ml during the first week of incubation, and after 3 weeks of incubation there were still 3×10^8 to 5×10^8 CFU/ml. Once this value was reached, and similar to what was observed after growth in LB, viable-cell counts remained very stable for many weeks. We have observed similar maintenance of viable-cell counts with a number of "wild-type" *E. coli* clinical isolates (D. Siegele and R. Kolter, unpublished observations).

In an effort to determine whether the reduction in viable-cell counts represents cellular death or a viable but not culturable state (35), we stained the cultures with the vital stain acridine orange (23). The fraction of live-staining cells versus dead-staining cells correlated well with the colony counts (results not shown). In addition, it was possible to separate the live-staining cells from the dead-staining cells by using Renografin gradients (8), and the CFU were found in fractions containing the live-staining cells. These results indicate that for ZK126 grown under the conditions described above, the CFU count accurately represents the viable cells in the culture.

Mutagenesis and screen for mutants unable to survive in stationary phase. To obtain "knock-out" mutations in genes not essential for growth but required for long-term survival, we used insertion mutagenesis with the defective transposon mini-Tn*10* (42). ZK126 was infected with λ::mini-Tn*10*, and random transposition events into the chromosome were selected by plating cells in the presence of kanamycin. When the colonies were still small, and therefore at least some of the cells still were dividing, they were toothpicked onto a grid pattern on plates containing kanamycin. After 8 h of growth, the colonies were used to inoculate two sets of microtiter dishes: one containing liquid medium and the other with the same liquid medium with 20% glycerol. The set of plates containing medium alone was incubated at 37°C for several days, while the set containing glycerol was kept at −70°C and served as a source from which to retrieve mutants which died after prolonged incubation. After several days of incubation at 37°C, the microtiter plates were tested for the presence of viable cells by plating samples onto medium containing kanamycin. Any well that did not show any viable cells represented an insertion mutant that was then retested for its inability to survive in stationary phase.

This type of screen has been performed after the cells have been grown in LB medium (this paper) and in M63 minimal salts plus glucose (D. Siegele and R. Kolter, unpublished results). After the initial screening of about 20,000 colonies from 100 independent transductions, there were 30 candidates displaying reduced viability. Upon rescreening, a total of four mutants were obtained, all of which carried mutations in different and previously uncharacterized genes. We designated these genes *sur* (for survival in stationary phase). This article contains the mapping and detailed characterization of the effects of one such mutation, initially identified in the LB screen, which we have designated *surA*. The mini-Tn*10* insertion into *surA* is designated *surA1*.

Viability of ZK126 *surA1*. Figure 1 shows the viability of

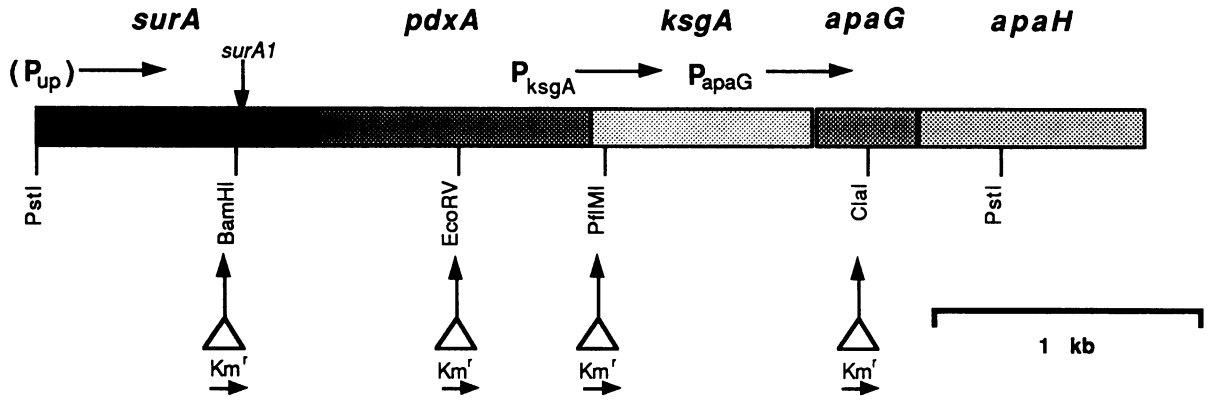

FIG. 2. Physical map of *surA* and downstream genes. The location of promoters identified previously is shown (34). Key restriction enzyme sites are indicated. The arrow under *surA1* points to the insertion site of the mini-Tn*10* Km^r element. The location of insertion and direction of transcription of the kanamycin resistance cassette are also shown.

ZK126 *surA1* during prolonged incubation in LB plus kanamycin (Fig. 1A) and in M63-glucose plus kanamycin (Fig. 1B) (this culture was supplemented with pyridoxine, a vitamin B_6 precursor; see below). The viable-cell counts reached after the end of exponential growth were roughly equal for both the mutant and the parent strains. In addition, the doubling times of the mutant strain in LB (20 min) and in M63-glucose (1 h) were also identical to those of ZK126 (results not shown). However, it is clear that the mutant strain was greatly defective in stationary-phase survival in both media, although the kinetics of death were much slower in the glucose-starved culture. Thus, the action of this gene product is not essential for growth in either of these media but is essential for maintenance of viability during stationary phase. As shown in Fig. 1A, the loss of viability observed in the mutant strain was complete ($<10^2$ CFU/ml), although suppressor mutations did arise; these are discussed below. To maintain the *surA1* strain, the original frozen stab was cultured to mid-log phase in LB. The culture was supplemented with 20% glycerol and was then divided into portions and stored at −70°C.

Mapping of *surA*. Cloning of the DNA fragment containing the insertion was accomplished by digesting chromosomal DNA with *Pst*I, a restriction enzyme that does not cut the mini-Tn*10* element, and ligating this DNA to *Pst*I-digested pUC19. Following transformation of ZK126 with this DNA, kanamycin-resistant recombinant clones were found to harbor a plasmid with a 5.45-kilobase-pair (kb) *Pst*I insert. Subtracting from this the 1.86 kb of the mini-Tn*10* element, the chromosomal *Pst*I fragment containing the insertion was presumed to be 3.59 kb. The site of insertion of the mini-Tn*10* element within this *Pst*I fragment was mapped by restriction enzyme digests and found to be 0.76 kb from one of the *Pst*I sites.

This recombinant plasmid was designated psurA1. Plasmid DNA was purified, labeled with [^{32}P]dATP by nick translation, and used as a probe on a filter containing the ordered λ library of *E. coli* W3110 (22). The probe hybridized to phages 104, 105, and 106 of the Kohara miniset, indicating that the *surA* gene is located near 1 min of the *E. coli* chromosome. Figure 2 shows the physical and genetic map of this region. The insertion occurred in an open reading frame just upstream of *pdxA*, a gene involved in the biosynthesis of pyridoxal phosphate (vitamin B_6) (34). This gene forms part of a complex operon which also contains *ksgA*, *apaG*, and *apaH* (2–4, 7, 16, 34). From the various publications describing these genes, we were able to compile the entire sequence from the *Bam*HI site near the *surA1* insertion site to the end of the operon. Indeed, *surA* had already been recognized as a region containing an open reading frame coding for the C-terminal 100 amino acids of an unknown protein. To gather additional information, we sequenced the 759 bp found in psurA1 between the *Bam*HI site and the proximal *Pst*I site. This information yielded an additional 253 amino acids of the *surA* product, but it also demonstrated that the *Pst*I site is found within the *surA* coding region and that the beginning of this complex operon lies further upstream.

Polarity of *surA1* on downstream genes. Once the map position of the *surA1* mutation had been unambiguously determined, it was possible to assess what effect the insertion of the mini-Tn*10* element had on the expression of downstream genes. Previous work defined at least three promoters transcribing various regions of this operon, and these are shown in Fig. 2 (34). Two of these promoters have been accurately mapped (*ksgAp* and *apaGp*) while the third, p_{up}, was inferred to be somewhere upstream of the *Bam*HI site within *surA*. Since the insertion in *surA1* occurred just downstream of this same *Bam*HI site, it was possible that the observed Sur^- phenotype of the *surA1* mutation was the result of polarity on the expression of downstream genes. Indeed, the *surA1* mutation had a polar effect on *pdxA* expression, because ZK126 *surA1* was phenotypically Pdx^-, i.e., the strain could not grow on minimal medium, but the auxotrophy could be relieved by the addition of the pyridoxal phosphate precursor pyridoxine. In contrast, *surA1* did not appear to exert polarity on *ksgA* expression, since ZK126 *surA1* remained sensitive to kasugamycin (knock-out mutations in *ksgA* yield a kasugamycin-resistant phenotype [4]). This was not entirely unexpected given the presence of an internal promoter within *pdxA* which transcribes *ksgA*.

The data presented in Fig. 1B indicate that the polarity of *surA1* on *pdxA* expression was not the primary reason for the observed loss of viability (Sur^- phenotype) in these mutants, because addition of pyridoxine to minimal medium did not relieve the Sur^- phenotype. This phenotype could not be reversed in LB cultures either, even when supplemented with excess pyridoxine. Regardless of these suggestive results, it was still necessary to directly determine the viability of strains mutated in the genes downstream of the operon.

To facilitate the construction of mutations in downstream

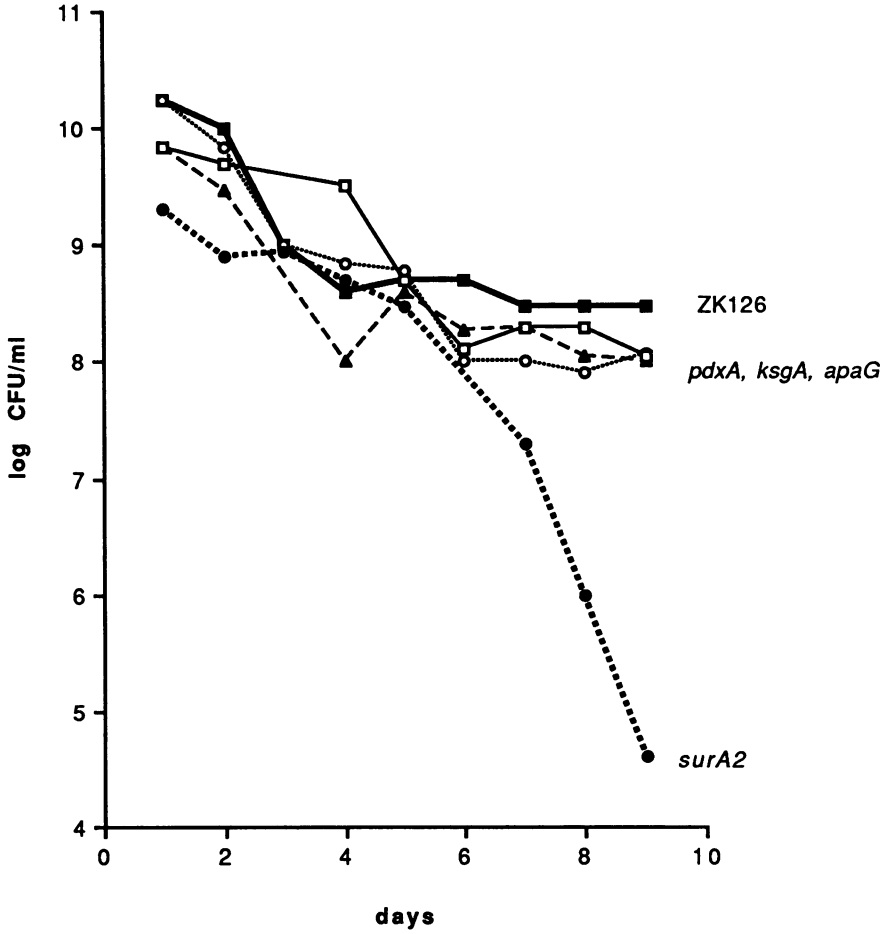

FIG. 3. Long-term survival of ZK126 derivatives harboring kanamycin resistance cassette insertions in four of the genes of the *surA* operon. The insertions into the four genes were generated as described in Materials and Methods. The strains were incubated in LB with kanamycin. Symbols: ■, ZK126; ○, ZK126 *pdxA*; ▲, ZK126 *ksgA*; □, ZK126 *apaG*; ●, ZK126 *surA2*.

genes, we first cloned the wild-type *Pst*I fragment into which the *surA1* insertion had occurred. This was greatly facilitated because the complete *pdxA* gene is found within this fragment and ZK126 *surA1* is phenotypically Pdx$^-$. This made it possible to isolate the wild-type clone by transforming ZK126 *surA1* with pUC19 ligated to a *Pst*I digest of ZK126 DNA and selecting for Amp^r and Pdx$^+$. We designated this recombinant plasmid psurA$^+$.

Once the wild-type fragment was obtained, unique sites within the coding regions of *surA*, *ksgA*, and *apaG* were used to insert a kanamycin resistance cassette into each of these genes in the ZK126 background (see Materials and Methods and Fig. 2). A similar insertion of a kanamycin resistance cassette into *pdxA* was obtained from M. Winkler (34). Thus, we obtained the following: a second allele of *surA* (*surA2*), and similar insertion mutations in three of the four downstream genes, *pdxA*, *ksgA*, and *apaG*. The viability of these strains was tested in LB plus kanamycin, and the results are shown in Fig. 3. It is apparent that the *surA2* (like the *surA1*) strain displayed the Sur$^-$ phenotype, while the other mutants displayed a normal Sur$^+$ phenotype. From these results it is possible to conclude that the Sur$^-$ phenotype is the result of the mutation in *surA* and not of polar effects on the expression of downstream genes.

Analysis of Sur$^+$ revertants. The results presented thus far have all involved cultures incubated in the presence of kanamycin. The results of an experiment in which ZK126 *surA1* was grown and kept in LB without kanamycin are shown in Fig. 4A. Dilutions of the culture were plated every day on several different media to determine the number of CFU in the culture having each of the following properties. Total viable-cell counts were determined by plating cells on LB alone. Colonies appearing on LB with kanamycin represent cells that still contain the mini-Tn*10* insertion. In contrast, colonies able to plate on M63-glucose without added pyridoxine represent cells in which the mini-Tn*10* has been excised (phenotypically Pdx$^+$). After 1 day in stationary phase, no cells ($<10^2$ CFU/ml) were phenotypically Pdx$^+$. This was interpreted as an indication that in this particular culture, no excisions of the mini-Tn*10* occurred during growth. While the overall viable-cell counts decreased to 5×10^7 CFU/ml during the first 4 days, by day 5 total viable-cell counts rose again to almost 10^9 CFU/ml. However, by day 6 there were only 10^4 CFU of Km^r cells per ml in the culture, and most of the cells in the culture were Pdx$^+$ Km^s. Apparently, one or more excisions of the mini-Tn*10* element occurred some time in the first 2 days of incubation. By the end of the second day, there were 10^3 CFU/ml with a Pdx$^+$ phenotype. These cells apparently grew "exponentially" until by day 6 they had almost completely taken over the culture.

The possibility remained that the Pdx$^+$ cells were not

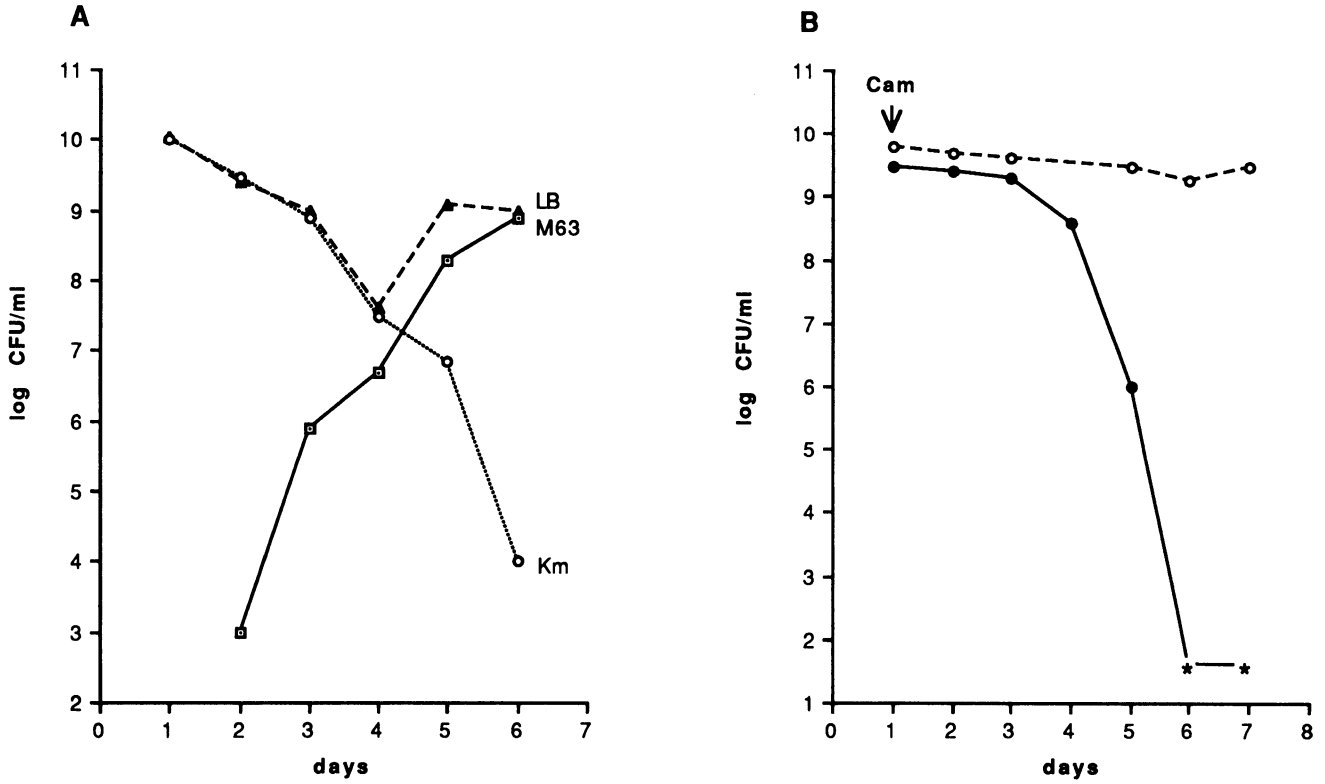

FIG. 4. Growth of Sur$^+$ revertants from a culture of ZK126 *surA1* grown in the absence of kanamycin. (A) A single culture of ZK126 *surA1* was followed by plating on LB (▲), LB with kanamycin (○), and M63-glucose (⊡). (B) Parallel cultures of ZK126 (○) and ZK126 *surA1* (●) were treated with 200 μg of chloramphenicol (Cam) per ml on day 1 (as indicated by the arrow), and their viability was followed by plating on LB. Stars indicate viable counts were $<10^2$ CFU/ml.

growing but rather were accumulating due to an increased frequency of precise excision during incubation in stationary phase. To rule this out, a culture of ZK126 *surA1* in LB was treated with the bacteriostatic antibiotic chloramphenicol after 1 day in stationary phase (Fig. 4B). While such treatment did not have any effect on the viable-cell counts of ZK126, the *surA1* mutant strain died completely. These results suggest that: (i) the *surA1* mutation did indeed cause the death of the cells in stationary phase and (ii) the Pdx$^+$ Km^s revertants (precise excisions and therefore Sur$^+$ as well) that appeared in LB-grown cultures were able to grow and take over the culture as the mutant cells died. The experiment presented in Fig. 4A was repeated five times with qualitatively similar results. The exact time of appearance of the first Pdx$^+$ cells varied, as expected if the time of excision of the mini-Tn*10* element fluctuates randomly.

The loss of the Km^r determinant in one of the Pdx$^+$ Sur$^+$ revertants was directly shown to be due to a precise excision. This was accomplished by cloning the Pdx$^+$ fragment by complementation of ZK126 *surA1* and analyzing the DNA with various restriction enzymes.

Suppressors of *surA1* in cultures grown in the presence of kanamycin. The results presented in Fig. 1A indicate that the culture of ZK126 *surA1* grown in the presence of kanamycin lost viability during the first 5 days of stationary phase. In repeating this experiment many times, we noticed that some cultures, even after reaching viable-cell counts below 10^2 CFU/ml, could grow back to 10^8 CFU/ml. Figure 5A shows the results of four separate experiments and demonstrates the fluctuation observed in the time of appearance of the Sur$^+$ suppressors. Here again, this fluctuation in the time of appearance is expected if the origin of the suppressor mutations is random. All Sur$^+$ suppressors analyzed to date were also phenotypically Pdx$^+$, indicating that suppressors most commonly arise by some form of precise or nearly precise excision. Thus, we were able to monitor the existence of Sur$^+$ cells not only by their ability to survive in stationary phase, but also by their ability to grow in the absence of pyridoxine.

We wanted to determine whether the Sur$^+$ Km^r suppressors preexisted in the culture (arose during exponential growth) or arose during incubation in stationary phase. To this end, cultures of ZK126 *surA1* were grown and kept in LB plus kanamycin and the appearance of Pdx$^+$ Km^r suppressors was monitored by plating on M63-glucose plus kanamycin. The results of one such experiment are shown in Fig. 5B. It is evident that no Pdx$^+$ cells arose during exponential growth and that once the first Pdx$^+$ Km^r cells appeared, they grew exponentially and took over the culture. In contrast to the Pdx$^+$ Km^s cells that appeared in cultures grown in LB alone, these Pdx$^+$ cells were not the result of simple excision events because they remained Km^r.

In an attempt to understand the nature of the events leading to the appearance of Sur$^+$ Pdx$^+$ Km^r suppressors, we cloned the Pdx$^+$ and the Km^r determinants from these cells into pUC19 by using *Pst*I. DNA was prepared from a culture of a colony-purified suppressor strain, digested with *Pst*I, and ligated to the plasmid vector, In one transformation, the recipient cells were ZK126 and selection was for kanamycin resistance. The resulting plasmids were physically identical to psurA1 and were designated psurA1-sup-Km^r. In a second transformation, the recipient was

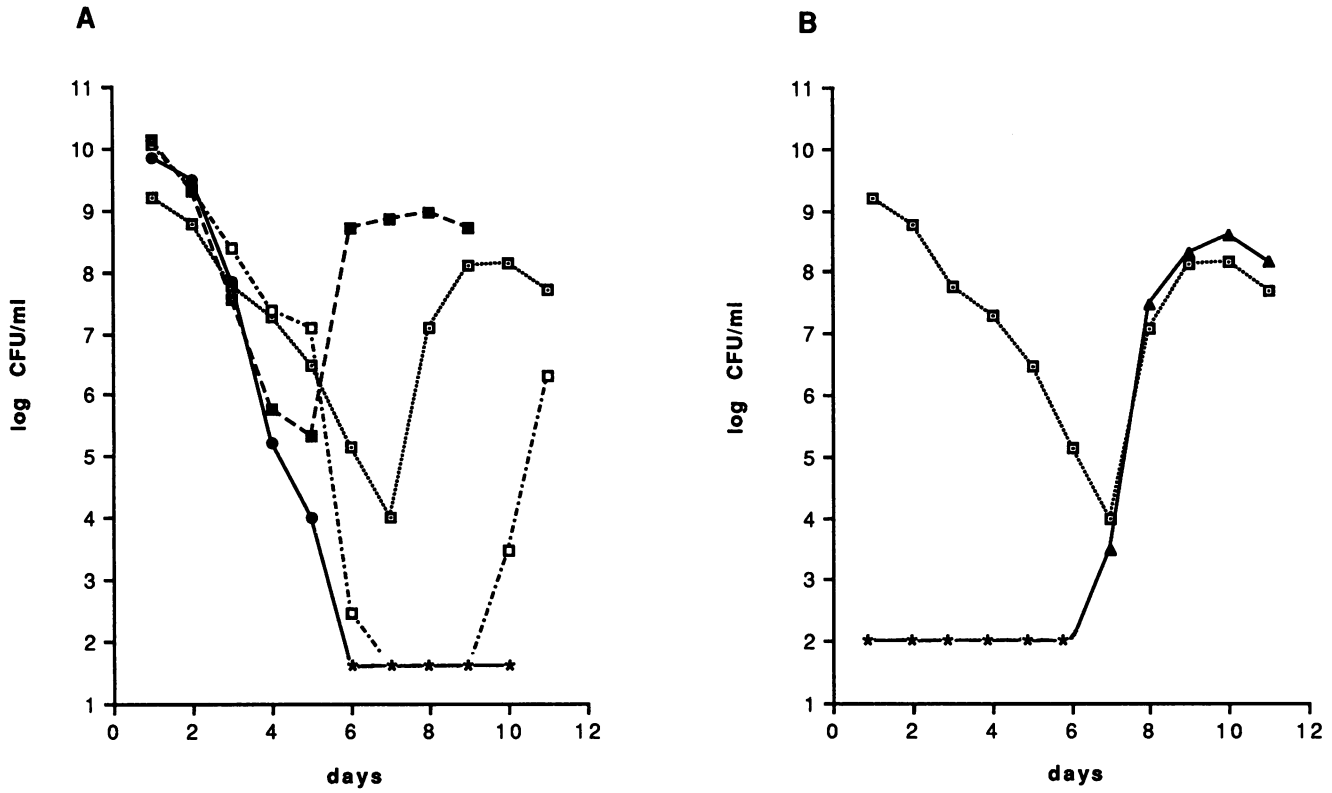

FIG. 5. Sur$^+$ Km^r suppressors of ZK126 *surA1*. (A) Fluctuation in the appearance of suppressors as a function of time. One culture that never showed suppressors is shown (●) along with three different cultures in which suppressors arose at various times. All cultures were incubated in LB with kanamycin and plated on LB with kanamycin. (B) A culture in which suppressors arose was monitored by plating on LB with kanamycin (□) and M63-glucose with kanamycin (▲). Stars indicate viable counts of $<10^2$ CFU/ml.

ZK126 *surA1* and selection was for Pdx$^+$. In this case, the resulting plasmids were physically identical to psurA$^+$ and were designated psurA$^+$-sup-Pdx$^+$. These results indicated that in the suppressor cells, both the wild-type *surA* gene and the mutant *surA1* allele were found.

To confirm this, *Pst*I-digested DNA from the suppressor cells was hybridized with labeled psurA1 as the probe (Fig. 6). It is clear that the suppressors must contain a duplication of the region containing *surA* and that there has been an excision of the mini-Tn*10* in one of the copies of *surA*. While we have no way of determining when the duplication occurred, the excision must have occurred between days 6 and 7 of incubation, when the viable-cell counts of the culture were between 10^5 and 10^4 CFU/ml (Fig. 5B). We sequenced the DNA in the Pdx$^+$ fragment to determine the nature of the excision of the mini-Tn*10* element. The excision was nearly precise; there was a loss of a C residue and the insertion of a G residue 1 base pair away. The result was a proline to glutamine codon change within the coding region of *surA*. When the suppressor strains were regrown and retested for their suppressor phenotype, they proved to be unstable. This observation is consistent with their being the result of gene duplications.

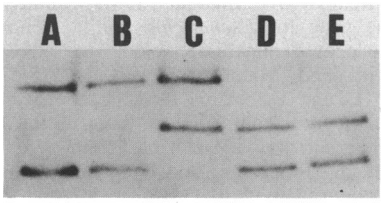

FIG. 6. Southern filter hybridization analysis of *surA1* suppressors. Analysis was performed with psurA1 as a probe. The lanes contained the following *Pst*I-digested DNAs: (A) psurA1, (B) psurA1-sup-Km^r, (C) chromosome from Sur$^+$ Pdx$^+$ Km^r suppressors, (D) psurA$^+$, and (E) psurA$^+$-sup-pdx$^+$. The lower band present in lanes A, B, D, and E is the pUC19 vector. The upper band present in lanes A, B, and C is the *Pst*I fragment containing *surA*, *pdxA*, *ksgA*, and *apaG* (see Fig. 2) with the mini-Tn*10* insertion. The middle band present in lanes C, D, and E is the wild-type *Pst*I fragment containing the same genes.

DISCUSSION

It is widely accepted that bacterial cells in the natural environment exist in constant flux between short periods of exponential growth and much longer periods of nongrowth. This has been termed the "feast and famine" existence of bacteria: when nutrients are available, bacteria can attain rapid growth rates, but when nutrients are depleted, they must be able to endure prolonged periods of starvation. Although it has been reported that certain strains of *E. coli* enter an irreversible exponential death phase upon phosphate starvation (13), in most "wild-type" strains that we have tested, a fraction of the initial viable cells are able to maintain the living state for extremely long periods of time.

Here we have presented results which, to our knowledge, represent the first isolation of an *E. coli* mutant specifically screened for its inability to survive prolonged periods of nutritional starvation. The ZK126 *surA1* mutant, in experiments in which suppressor mutations did not arise, died

exponentially and completely within a week of the onset of stationary phase when grown in rich medium (Fig. 1A). Mutant strains of *E. coli* and *Salmonella typhimurium* containing starvation-inducible Mu d*lac* fusions have been reported to lose viability at higher rates than the parent strains, but the death of these strains was not complete (17, 40).

As indicated in the Introduction, many products are known whose synthesis is increased at the onset of stationary phase. Their role in maintenance of the viable state, however, remains uncertain. The finding of genes which are not essential for growth in exponential phase but are required for survival in the absence of growth provides a new way to analyze the essential physiological processes active only during stationary phase.

The mutation described in this article, *surA1*, defines a new gene in *E. coli*. This gene is the furthest-upstream gene known within a complex operon which contains *pdxA*, *ksgA*, *apaG*, and *apaH* (34). It is not yet known whether there are additional genes upstream of *surA*. The insertion in *surA1* is polar on the expression of *pdxA*, rendering the mutants phenotypically Pdx^-. This phenotype, however, is separable from the Sur^- phenotype, as indicated by these two results: (i) addition of pyridoxine did not cause reversion to the Sur^- phenotype and (ii) a *pdxA* mutant did not display the Sur^- phenotype.

In an article describing the transcription of *pdxA* (34), RNA originating from a promoter located somewhere upstream of the *Bam*HI site shown in Fig. 2B (p_{up}) was detected during exponential growth. Whether this RNA represents a transcript of the entire *surA* coding region or initiates within *surA* is not known. It will be interesting to determine whether *surA* is transcribed during growth or only during stationary phase. Experiments to determine the regulation of transcription of *surA* are in progress in our laboratory.

A striking finding from the results presented here is the dynamic state of cultures during stationary phase. The time of appearance of revertants and suppressors of *surA1* indicates that DNA rearrangements can occur in populations containing remarkably small numbers of viable cells. Assuming that suppressors can grow at a constant rate, it is possible to extrapolate back to the time of appearance of the first suppressor in each culture shown in Fig. 5A and estimate the number of viable cells present in that culture at that time. In one culture the suppressor arose during the fourth day, with about 10^6 CFU/ml remaining. In another culture the event occurred on the sixth day, when the viable-cell counts were 10^5 CFU/ml. In yet another the suppressor appeared at about day 9, when there were less than 10^2 CFU/ml in the culture. Thus, the frequency of appearance of suppressors per bacterium-hour actually increased with prolonged incubation time (Fig. 5A). This unexpected acceleration in the mutation rate during nutritional deprivation has also been observed in the excision of Mu phage and insertion sequences from the chromosome (9, 18, 29, 38). Once these rearrangements have occurred, the resulting cells are able to grow and take over the cultures.

The identification of a gene whose function is essential only during starvation suggests that some metabolic processes operate only during stationary phase. Indeed, the existence of such processes in bacteria was already suggested by the isolation of a *Rhizobium meliloti* mutant unable to utilize pyruvate, acetate, or arabinose but still metabolized [^{14}C]pyruvate during stationary phase and released $^{14}CO_2$ (15). Knowledge of these mechanisms may help us understand the unexpected results obtained by Shapiro (38), Cairns et al. (9), and Hall (18) in analyzing the origin of mutations in stationary phase. In some of these reports, directed mutagenesis and adaptive evolutionary mechanisms were invoked to explain the selective appearance of mutations in aging cultures of *E. coli*. In short, during stationary phase the frequency of mutations from Lac^- to Lac^+ (or Sal^- to Sal^+) was specifically increased by the presence of the nonutilizable carbon source in the medium. An alternative explanation was offered in which the induction of transcription by the presence of the inducer exposed the single-stranded DNA more readily to mutagenesis (12). This theory came short in terms of explaining where the energy to carry out the metabolic processes of transcription and mutagenesis might come from in starved cultures. Results presented here suggest that there may be heretofore unknown metabolic processes which could provide the energy in nongrowing cells.

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