

competent for retention of McpA. Although the presence of the McpA chemoreceptors at the stalked pole is due to lack of turnover, it may be that under normal circumstances the newly synthesized McpA is targeted to the stalked pole as well as to the flagellated pole, but is degraded at the stalked pole soon after synthesis. We know that in *E. coli* the chemoreceptors can be targeted to both cell poles (13). Thus, proteolysis could play a role in the spatial distribution of McpA in *C. crescentus* by removing McpA from the stalked pole of the predivisional cell. Perhaps the presence of protease at the stalked cell pole prevents the deposition of other proteins that are used for the assembly of the flagellum and pili. There is evidence for spatially restricted proteolysis in eukaryotic cells. Localized proteolysis has been shown to be involved in setting up restricted protein distribution in polarized epithelial cells, resulting in the removal of proteins from one membrane domain and not the other upon induction of polarization (14).

The specific degradation of McpA could be mediated by a localized activity that modifies the protein, rendering the polypeptide susceptible to degradation by a protease that is present in all cells. Alternatively, the protease could be present or specifically activated only in the stalked cell. A stalked cell-specific protease is likely to be cytoplasmic or possibly associated with the inner membrane, because the COOH-terminus of the chemoreceptor is in the cytoplasm. There is evidence that the cytoplasmic Lon protease is involved in *Myxococcus xanthus* fruiting body formation (15). It has been shown that the *C. crescentus* homologue of the Lon protease preferentially segregates to the stalked cell upon division of the predivisional cell (16). The fact that Lon segregates to the stalked cell, and not to the swarmer cell, suggests that it might be involved in degradation of any McpA that ends up in the stalked cell portion of the predivisional cell. It is not yet known whether Lon recognizes McpA and whether Lon is specifically targeted to the stalk pole.

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- pJM21, pJM22, and pJM23. These vectors have the M2 epitope in three different reading frames with respect to the polylinker. They also contain an in-frame stop codon with respect to the M2 epitope, and this codon is followed by the site for the restriction enzyme Spe I. These constructs were transferred on Eco RI–Spe I fragments from the M2 epitope vectors into a plasmid capable of replication in *C. crescentus*, pRK290KS1 (2). There was no effect attributable to the copy number of the plasmid, because the McpA protein derived from the plasmid-borne *mcpA* gene on pRCH9 (three to five copies per cell) showed a pattern of cell-cycle turnover similar to that of a single chromosomal copy of *mcpA* (Fig. 1).
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19. McpA is not synthesized in swarmer cells but is synthesized later in the cell cycle (2). Total protein synthesis continues during transition from the swarmer to stalked cell. Therefore the ratio of McpA to total protein will decrease prior to McpA synthesis later in the cell cycle. By loading an equal volume of culture in each lane, we avoided observing a decrease in McpA because of the lack of its synthesis during the early part of the cell cycle.
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23. The *mcpA* deletion carried on plasmid pRCM223 (Fig. 3) was introduced into a synchronizable derivative of *C. crescentus* CB15, NA1000, and cell cycle immunoblots were performed on this strain with antiserum to McpA, as described in the legend of Fig. 1. Therefore, a direct comparison of the stability of the wild-type and deleted proteins could be performed in the same immunoblot.
24. Supported by Public Health Service grant GM 32506 (L.S.) and an NIH postdoctoral fellowship GM13929 (J.R.M.). We would like to thank Dale Kaiser and colleagues from the Shapiro laboratory for critical reading of the manuscript.

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## Microbial Competition: *Escherichia coli* Mutants That Take Over Stationary Phase Cultures

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Many microorganisms, including *Escherichia coli*, can survive extended periods of starvation. The properties of cells that survived prolonged incubation in stationary phase were studied by mixture of 10-day-old (aged) cultures with 1-day-old (young) cultures of the same strain of *Escherichia coli*. Mutants from the aged cultures that could grow eventually took over the population, which resulted in the death of the cells from the young cultures. This phenotype was conferred by mutations in *rpoS*, which encodes a putative stationary phase-specific sigma factor. These rapid population shifts have implications for the studies of microbial evolution and ecology.

Bacteria can remain viable under conditions of poor nutrient availability. Many microorganisms respond to starvation by forming dormant spores, which are generally resistant to extreme environments (1). Nonsporulating Gram-negative bacteria, among them *Escherichia coli*, remain metabolically active but also develop increased resistance to a variety of environmental stresses after exponential growth has

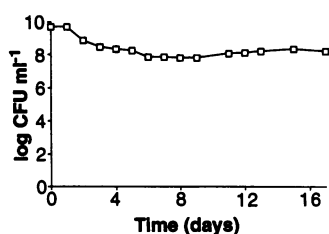
stopped and cells enter stationary phase (2). In Gram-negative bacteria, the overall rate of protein synthesis decreases, but distinct sets of proteins are induced upon entry into stationary phase (3, 4). Some of these proteins protect the cell against environmental challenges such as oxidative damage; others are necessary to maintain viability (5, 6). The molecular mechanism of this response involves the induction of at least one regulon, defined by the genes whose expression depends on the putative stationary phase-specific sigma factor  $\sigma^S$ , the product of the *rpoS* gene also known as *katF* (4, 7). In this report we show that mutations in *rpoS* can have profound effects on the ability of cells to compete and

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**Fig. 1.** Viability of *E. coli* ZK126 in LB. A 3-ml culture was kept aerated on a roller at 37°C.

survive under certain conditions in stationary phase cultures.

We studied *E. coli* survival in stationary phase. Our standard wild-type *E. coli* strain (ZK126) saturates at  $\sim 1.0 \times 10^9$  to  $2.0 \times 10^9$  colony-forming units (CFU) ml<sup>-1</sup> when grown in M63 minimal medium with 0.2% glucose. The number of viable counts remains constant for many days and even weeks under these conditions (8). The same strain saturates at a density of  $\sim 0.5 \times 10^{10}$  to  $1.0 \times 10^{10}$  CFU ml<sup>-1</sup> when grown in rich LB medium (Fig. 1). Viability over the first week decreased by one or two orders of magnitude, after which the viable counts stabilized at  $\sim 10^8$  CFU ml<sup>-1</sup>.

Cells of the ZK126 strain that were cultured in LB were examined microscopically at different times during 10 days of incubation. To distinguish between live and dead cells, methanol-fixed samples were stained with acridine orange (8). Once stained, LB-grown ZK126 cells fluoresce orange if alive and green if nonviable. As expected, exponential phase cells were rod-shaped and fluoresced orange; cells that entered stationary phase became spherical (9) and fluoresced orange (Fig. 2A). After 3 days of incubation, most of the cells had lost viability when assayed for CFU (Fig. 1) and fluoresced green (Fig. 2B). By day 10, the fraction of surviving cells had once again become elongated (Fig. 2C). To determine if these cells were undergoing cell division, 10-day-old cultures were treated with the antibiotic aztreonam, which inhibits septation but not cell elongation (10). Long filaments were observed in a 10-day-old, but not a 1-day-old, culture treated with aztreonam (Fig. 2D), which indicates that cell division was indeed taking place in these cultures.

To determine how the survivors from a 10-day-old (aged) culture behaved when reintroduced into a 1-day-old (young) culture, we mixed cells from both cultures and determined the numbers of each over several days. Cells from young and aged cultures were distinguished by either nalidixic acid (*Nal*<sup>R</sup>) or streptomycin (*Sm*<sup>R</sup>) resistance markers. To avoid any possible detrimental effect of the 10-day-old medium on the young culture, a small sample (3  $\mu$ l) from an aged culture of ZK126 *Sm*<sup>R</sup>

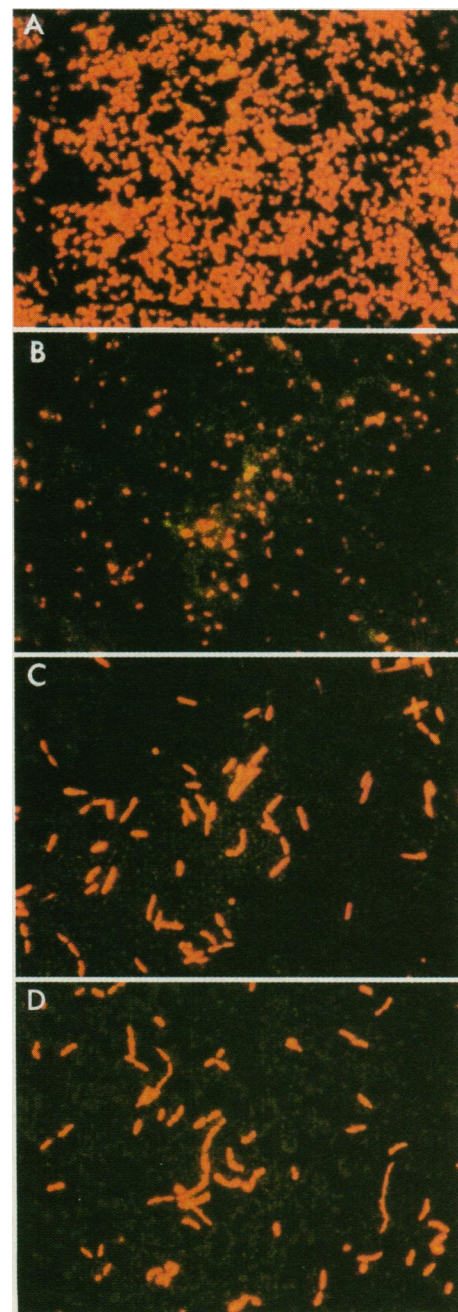
was mixed with a young culture of ZK126 *Nal*<sup>R</sup> (3 ml). The mixed culture was incubated for 2 weeks, and we determined the viable counts of each population at various times by plating on appropriate media (Fig. 3A). In these mixed cultures, cells from the aged culture grew and took over the population, with a concomitant loss of viable cells from the young culture. When cells from young and aged cultures were mixed in equal numbers ( $\sim 10^6$  CFU ml<sup>-1</sup> each) in fresh LB liquid medium, both cell populations saturated at  $\sim 5 \times 10^9$  CFU ml<sup>-1</sup>. Again, cells from the young culture lost viability whereas cells from the aged culture survived (Fig. 3B). Thus cells from aged *E. coli* cultures had a competitive advantage in stationary phase because they could both grow and cause the death of cells from a young culture. This phenotype was observed regardless of which population carried the *Nal*<sup>R</sup> or *Sm*<sup>R</sup> marker, which indicates that this phenomenon is not due to the presence of a particular antibiotic resistance marker. A strain that lacks any antibiotic resistance marker gave similar results (12). This phenotype was expressed only in stationary phase and did not affect exponentially growing cells (Fig. 3B). When cells from two young cultures were mixed, the cells in the minority did not grow and often died out slowly (Fig. 3C).

One possible explanation for the death of cells from the young culture could be that the cells from the aged culture release a stable toxic product or an antibacterial agent that accumulates in stationary phase. However, this hypothesis was disproved because cells from a young culture that were resuspended in filter-sterilized medium from a 10-day-old culture remained viable. Alternatively, death of the young culture cells could result indirectly from competition with cells better able to survive under these particular starvation conditions.

After repeated cycles of exponential growth in liquid or solid medium, cells from aged cultures could still take over those from young cultures (13). We obtained several strains that always expressed the phenotype by isolating cells directly from aged cultures or after they had been mixed with and taken over the young population in mixed cultures. This stable inheritance suggests that the phenotype was due to a mutation or mutations and not to a reversible physiological adaptation.

Because *rpoS* participates in regulating stationary phase phenomena, we tested the strains with a growth advantage in stationary phase for mutations in that gene. One of the genes in the  $\sigma^S$  regulon is *katE*, which encodes the enzyme hydrogen peroxidase II (HPII) (6). The allelic state of *rpoS* can be examined indirectly by analysis of

HPII activity. This can be done semiquantitatively by addition of H<sub>2</sub>O<sub>2</sub> to bacterial colonies grown on LB plates. HPII breaks down H<sub>2</sub>O<sub>2</sub>, and the concomitant release of O<sub>2</sub> results in bubbling of the colony. Strains bearing the wild-type *rpoS* allele bubble vigorously, whereas strains bearing null alleles (for example, *rpoS::kan*) bubble only



**Fig. 2.** Acridine orange-stained samples from LB cultures. Samples taken from (A) 1-day-old, (B) 3-day-old, and (C) 10-day-old cultures were spotted onto microscope slides, fixed with methanol, and stained with acridine orange (8). Stained cells were viewed under a Zeiss fluorescence microscope with a 487709 filter. (D) Cultures (10-day-old) were treated with aztreonam (0.1  $\mu$ g ml<sup>-1</sup>) (10) for 48 hours and then stained with acridine orange.



slightly (14). When the strains that expressed the phenotype of stationary phase growth advantage were treated with  $H_2O_2$ , many, but not all, displayed an intermediate bubbling phenotype, which suggests the presence of a mutation in *rpoS*.

Genetic mapping experiments with P1 transduction showed that the linkage between several mutations that result in reduced bubbling and *cysC* was similar to that between *rpoS* and *cysC* [45% cotransducible (14)]. The mutations also led to a reduction in the expression during stationary phase of the *rpoS*-dependent *bolA::lacZ* fusion (14). These results were all consistent with the hypothesis that many strains that expressed the stationary phase growth advantage phenotype harbored a mutation in *rpoS*. The location of one such *rpoS* mutation (designated with the allele number *rpoS819*) was confirmed by marker rescue with a fragment that contained the 3' half of the *rpoS* gene and by DNA sequencing.

The wild-type *rpoS* and *rpoS819* alleles were cloned and sequenced (15), revealing a 46-base pair duplication at the 3' end of the *rpoS819* gene (16). The mutant and wild-type proteins are identical up to four amino acids from the end. At this point the duplication in the *rpoS819* gene causes a frame shift that replaces the final four amino acids with 39 new residues (Fig. 4). The additional amino acids lie close to the helix that is thought to recognize the "−35" region of promoters (17). Polymerase chain reaction amplification of the 3' region of mutated *rpoS* alleles from different strains revealed that not all of these putative mutations have the same sequence change (18).

To determine whether the *rpoS* mutation alone caused the growth advantage phenotype, we transduced the *rpoS819* allele, by way of its linkage to *cysC*, from a strain expressing the phenotype into wild-type cells. We determined the presence of the *rpoS819* allele by assaying for bubbling of cells upon treatment with  $H_2O_2$ . The resulting strain was grown and, after 1 day in stationary phase, tested by mixture both as a minority in a stationary phase culture (Fig. 3D) or in equal numbers in fresh medium (Fig. 3E) with cells bearing the wild-type *rpoS* allele. The *rpoS819* allele conferred the stationary phase growth advantage phenotype on an otherwise wild-type strain. Similar results were obtained with another *rpoS* allele, *rpoS58* (19). In contrast, strains that contained *rpoS* null alleles did not express the stationary phase growth advantage phenotype but died rapidly when mixed with strains that retained *rpoS* function.

Having determined that transduction of certain *rpoS* mutant alleles into wild-type cells confers a growth advantage in stationary phase over unchanged wild-type cells, we tested whether additional mutations could confer a similar growth advantage over cells that contained the mutant *rpoS* alleles. Cells from young and aged cultures of *rpoS819* strains were mixed and cells from aged cultures again grew and caused the death of the young culture (Fig. 3F). This second cycle of aging led to a growth advantage in stationary phase that resulted from a second, unlinked mutation. This mutation requires the presence of the *rpoS819* allele to express its growth advantage phenotype. We have obtained a trans-

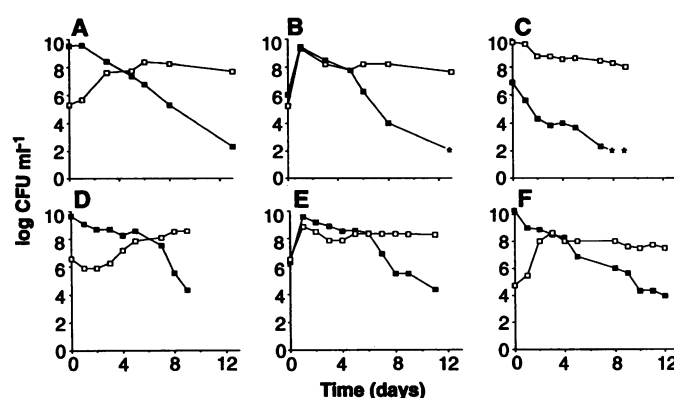
poson insertion linked to this second mutant locus, near minute 73 of the *E. coli* chromosome, but the mutant gene has not yet been identified (20). We have also obtained a mutation, near minute 27, that renders cells resistant to killing by cells from an aged culture.

During prolonged incubation, mutants with a competitive advantage replace the original population under the strong selective pressure imposed by starvation. These population takeovers, which occurred more rapidly than the population shifts reported for growing *E. coli* cultures (21), have implications for the study of the origin of mutations in starved microorganisms. Several reports suggest that, in stationary phase cultures, mutations occur more often when advantageous and so are a direct response to particular environmental challenges (22). However, it has often been assumed that stationary phase colonies or cultures are static or nearly static. The observation that stationary phase cultures are dynamic raises the possibility that many of the "post-selection" mutations that have been reported could have arisen from a minority of preexisting mutant cells that were able to grow in the presence of starved cells without changing the overall bacterial counts. Interpretations regarding the appearance of mutations in stationary phase cultures should therefore take this possibility into consideration (23).

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11. LB cultures (3 ml) were incubated in 18 mm by 150 mm glass test tubes and kept aerated by rotation in a New Brunswick roller at 37°C. Mixes (Fig. 3, A, C, D, and F) were done by transfer of 3  $\mu$ l of the culture as a minority into the young culture. Cocultures (Fig. 3, B and E) were done by mixture of approximately equal numbers of CFU in fresh LB medium (3 ml). We determined viable cell counts by making serial dilutions in M63 salts

**Fig. 3.** Mixed culture experiments (11) conducted with (A) cells from an aged culture (□) in the minority and cells from a young culture (■) in the majority, (B) cells from aged (□) and young (■) cultures in equal concentrations, (C) cells from two young cultures, (D) and (E) *rpoS819* (□) and wild-type *rpoS* cells (■), and (F) two *rpoS819* strains in which cells from an aged culture (□) are in the minority and cells from a young culture (■) are in the majority. Asterisks indicate that no colonies were detected at the lowest dilution plated (10  $\mu$ l directly from the culture).



**Fig. 4.** Comparison of COOH-terminal amino acid sequences of the wild-type and *rpoS819* gene products. A 46-base-pair duplication in the *rpoS819* gene resulted in the replacement of the last four residues in  $\sigma^S$  with 39 new amino acids. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

$\sigma^S$  : . . . IEA LFRF  
 $\sigma^{819}$  : . . . IEA PFARNPANAGAEYRSAPVPRVSKHLSEPVSSSEAGLFCAQ

- and plating onto both LB-Sm and LB-Nal plates. The media we used have been described [J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972)].
12. Cells from an aged ZK126 culture were mixed with a young ZK126 *Nal<sup>R</sup>* culture. Dilutions made in M63 were plated on LB and LB Nal media to determine the total number of viable organisms and the number containing the *Nal<sup>R</sup>* marker, respectively. After 8 days, the number of *Nal<sup>R</sup>* CFU per milliliter dropped from  $10^9$  to  $10^6$ , which indicates that cells from the aged ZK126 culture were taking over the population.
  13. Aged cultures and cells that had overtaken young cells in a mixed culture were streaked out on LB plates with the appropriate antibiotic. Isolated colonies were then grown in liquid LB for 1 day. We then tested these cultures by mixing them as a minority with young cultures.
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  15. To clone the wt *rpoS* and *rpoS819*, Kpn I-digested chromosomal DNA was ligated to pUC19 and used to transform a strain bearing the *rpoS::kan* mutation. Because cells develop an *rpoS*-dependent resistance to low pH [P. L. C. Small and S. Falkow, *ASM Abstr.* B74, 38 (1992)], ampicillin-resistant transformants (selected at 30°C) were incubated for 30 min in LB (pH 2.5) to select for plasmids that harbor the *rpoS* gene. Survivors were then screened for the *rpoS* gene by restriction enzyme analysis of plasmid DNA. Plasmid DNA was sequenced with Sequenase (U.S. Biochemical) and primers were synthesized on the basis of the published sequence of *rpoS* (7).
  16. Our sequences of the wt and mutant alleles of *rpoS* have been submitted to GenBank (accession number X16400). The sequence of the wt allele differed slightly from a previously published sequence (7); the differences have been noted in the GenBank entry. The most significant change is the absence of a base near the end of the coding region (position 1020), which shortens the predicted protein product by 20 amino acids from its originally reported length. When we sequenced this region from both the *rpoS* gene obtained by Mulvey and Loewen (7) and the same gene from our strain, ZK126, we found them to be identical.
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  18. DNA was amplified from whole-cell extracts with the use of AmpliTaq polymerase (Perkin-Elmer) and the following primers: 5'-GTTAACGACCAT-TCTCG-3' and 5'-TCACCCGTGAACGTGTC-3'.
  19. Many laboratory strains of *E. coli* K-12 show reduced amounts of *rpoS*-regulated genes, which suggests that these strains may have inadvertently undergone selections similar to those of our experiments.
  20. The defective transposon mini-Tn10kan [J. C. Way, M. A. Davis, D. Morisato, D. E. Roberts, N. Kleckner, *Gene* 32, 369 (1984)] was used to generate random transposition events in the chromosome of *rpoS819* cells that were isolated from an aged culture. Phage P1 was grown on a pool of the kanamycin-resistant (*Km<sup>R</sup>*) cells, and the lysate was used to transduce *rpoS819* cells to *Km<sup>R</sup>*. *Km<sup>R</sup>* transductants were grown in LB for 1 day and mixed as a minority with a young *rpoS819* culture. Cells that grew from the minority population were isolated and used to determine the linkage between the stationary phase growth advantage phenotype and the *Km<sup>R</sup>* marker (50% cotransducible). We mapped the mini-Tn10 insertion by cloning the *Km<sup>R</sup>* marker in pUC19 and using this plasmid as a hybridization probe against filters that were blotted with Kohara's ordered phage library of the *E. coli* chromosome [Y. Kohara, K. Akiyama, K. Isono, *Cell* 50, 495 (1987)].
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## An Osmosensing Signal Transduction Pathway in Yeast

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Yeast genes were isolated that are required for restoring the osmotic gradient across the cell membrane in response to increased external osmolarity. Two of these genes, *HOG1* and *PBS2*, encode members of the mitogen-activated protein kinase (MAP kinase) and MAP kinase kinase gene families, respectively. MAP kinases are activated by extracellular ligands such as growth factors and function as intermediate kinases in protein phosphorylation cascades. A rapid, *PBS2*-dependent tyrosine phosphorylation of *HOG1* protein occurred in response to increases in extracellular osmolarity. These data define a signal transduction pathway that is activated by changes in the osmolarity of the extracellular environment.

Cell growth requires the uptake of water, driven by an osmotic gradient across the plasma membrane. When the external osmolarity increases, many eukaryotic cells are capable of osmoregulation by increasing their internal osmolarity (1). The molecular mechanisms used by eukaryotic cells to sense changes in external osmolarity and transduce that information into an osmoregulatory response are poorly understood. The yeast *Saccharomyces cerevisiae* responds to increases in external osmolarity by increasing glycerol synthesis and decreasing glycerol permeability, thereby accumulating cytoplasmic glycerol up to molar concentrations (2, 3).

We isolated osmoregulation-defective mutants of yeast (4) by first screening mutagenized cells for the failure to grow on high-osmolarity medium [YEPD (1% yeast extract, 2% bactopectone, 2% dextrose) supplemented with 0.9 M NaCl or 1.5 M sorbitol]. Mutants that grew well on YEPD but not on high-osmolarity medium (*Osm<sup>S</sup>*) were then assayed for cellular glycerol accumulation 1 hour after the addition of 0.4 M NaCl to the medium (3). *Osm<sup>S</sup>* mutants were all recessive and fell into one of four complementation groups, identifying four HOG (high osmolarity glycerol response)

genes, *HOG1* to *HOG4*. Of this collection of mutants, we further analyzed two mutants, *hog1-1* and *hog4-1*. The reduced glycerol response and *Osm<sup>S</sup>* of *hog1-1* and *hog4-1* cosegregated 2:2 in tetrads resulting from a backcross to wild type and are thus the result of a single mutation.

Genomic DNA fragments were cloned (5) that complemented the *Osm<sup>S</sup>* phenotype of *hog1-1* and *hog4-1*, respectively. To locate *HOG1* and *HOG4* on each genomic clone, we generated subclones and tested for complementation of the *Osm<sup>S</sup>* phenotype of the respective *hog* mutant (6). The chromosomal locus of each clone was marked with a selectable marker and shown to be tightly linked to the original *hog* mutation (7), demonstrating that *HOG1* and *HOG4* (or closely linked genes) had been cloned.

The nucleotide sequence of the *hog1-1*-complementing DNA (8) revealed that *HOG1* (GenBank accession number L06279) is a member of the MAP (mitogen-activated protein) kinase family (9). The *HOG1* sequence contains a single, large open reading frame of 1.2 kb encoding a 416-amino acid protein with a molecular size of 47 kD. Northern (RNA) blot hybridization with the cloned *HOG1* gene as probe revealed a 1.4-kb transcript whose abundance was unaffected by exposure of the cells to increased osmolarity. Near the *NH<sub>2</sub>*-terminus of the predicted amino acid sequence of *HOG1*, a stretch of 300 amino acids contains each of the strongly conserved amino acids found in protein kinases (10). This sequence is most similar to that of MAP kinase family members (11, 12),

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## Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures

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