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Genetic Evidence for Parallel Pathways of Chaperone Activity in the Periplasm of *Escherichia coli*

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The periplasm of *Escherichia coli* contains many proteins proposed to have redundant functions in protein folding. Using depletion analysis, we directly demonstrated that null mutations in *skp* and *surA,* as well as in *degP* and *surA,* result in synthetic phenotypes, suggesting that Skp, SurA, and DegP are functionally redundant. The Δ*skp surA*::*kan* combination has a bacteriostatic effect and leads to filamentation, while the *degP*::Tn*10 surA*::*kan* combination is bactericidal. The steady-state levels of several envelope proteins are greatly reduced upon depletion of a wild-type copy of *surA* in both instances. We suggest that the functional redundancy of Skp, SurA, and DegP lies in the periplasmic chaperone activity. Taken together, our data support a model in which the periplasm of *E. coli* contains parallel pathways for chaperone activity. In particular, we propose that Skp and DegP are components of the same pathway and that SurA is a component of a separate pathway. The loss of either pathway has minimal effects on the cell, while the loss of both pathways results in the synthetic phenotypes observed.

In *Escherichia coli* proteins are targeted to four distinct cellular locations: two very different aqueous environments, the cytoplasm and the periplasm; and two distinct membranes, the inner membrane and the outer membrane. The outer membrane serves as a barrier that protects the cell from its external environment. Generally speaking, this membrane contains three types of proteins: lipoproteins; surface organelles, such as pili; and the β-barrel proteins.

The folding of the β-barrel proteins is not well understood. These proteins are initially synthesized in the cytoplasm with an N-terminal signal sequence that directs them to the secretion machinery at the inner membrane (30). After translocation from the cytoplasm and signal sequence cleavage, the path that the β-barrel proteins follow en route to the outer membrane is unclear. However, there is a good deal of evidence which supports a periplasmic intermediate model in which the β-barrel proteins pass through the periplasm in soluble form before localizing to the outer membrane (for a review see reference 5), where many of them, such as LamB and OmpF, serve as pores through which solutes can diffuse into the cell.

To date, several groups of periplasmic factors have been implicated in the folding and targeting of various extracytoplasmic proteins. These include factors involved in the formation and isomerization of disulfide bonds, peptidyl-prolyl *cis-trans* isomerases (PPIases), and chaperones. The formation of appropriate disulfide bonds in the oxidizing environment of the periplasm is critical for proper folding of many noncytoplasmic proteins (for a review see reference 24). However, disulfide bond formation is not required for proper folding of the porins LamB and OmpF.

The PPIases are a group of proteins that are conserved in all organisms. In vitro, the PPIases are known to facilitate the *cis-trans* conversion of proline residues, a rate-limiting step in protein folding (for a review see reference 23). Thus far, four PPIases have been identified in the periplasm of *E. coli*: SurA, PpiA, FkpA, and PpiD (7, 13, 17, 19, 22, 25). Of these four proteins, SurA and PpiD are the only two for which there is any direct evidence for a role in folding of the β-barrel proteins. *surA* null strains exhibit phenotypes indicative of outer membrane perturbations. These phenotypes include mucoid colonies on plates and hypersensitivity to detergents, certain antibiotics, and hydrophobic dyes (17, 25). Perhaps even more intriguing are the folding defects of the porins LamB and OmpF seen biochemically. *surA* null strains have been shown to accumulate folded monomeric forms of both proteins in certain strain backgrounds (25). More recently, PpiD has been implicated in folding. Null mutations of *ppiD* have been reported to confer a synthetic phenotype in *surA* mutants (7). This indicates that these two parvulin-type PPIases have functional redundancy. Furthermore, cells lacking *ppiD* have altered outer membrane profiles (7). Reduced levels of outer membrane proteins could be the result of a folding defect.

Periplasmic chaperones are another group of proteins that could play a role in the targeting of outer membrane proteins. DegP has been shown to switch between chaperone and protease activities in a temperature-dependent manner (29). Specifically, in vitro folding assays have shown that DegP acts with chaperone activity on the substrates MalS and citrate synthase (29). Several groups of workers have obtained evidence that the small periplasmic protein Skp has chaperone activity (2, 3, 8). Skp was purified by Chen and Henning (3) on the basis of its ability to bind to unfolded OmpF in an affinity chromatography assay. Similar chaperone activity was assigned to Skp on the basis of phage display (2). Most recently, in vitro evidence has assigned a chaperone activity to SurA independent of its role as a PPIase (1).

Because the effect of null mutations in any one of the suspected periplasmic folding factors on targeting of outer membrane proteins is minimal, we believe that there is functional redundancy in the periplasm of *E. coli*. To address this possi-

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TABLE 1. *E. coli* strains

Strain	Relevant characteristics	Source or reference
MC4100	F$^-$ *araD139* Δ*(argF-lac)U169 rpsL150 relA1 flb5301 deoC1 thiA1 ptsF25 rbsR*	28
CLC328	MC4100 Δ*skp zae-502*::Tn*10*	4
AR236	MC4100 Δ*skp zae-502*::Tn*10*	This study
AR208	MC4100 *surA*::*kan*	This study
AR299	AR236 *ara*$^+$	This study
AR394	AR299/pAER1	This study
AR412	AR394 *surA*::*kan*	This study
JMR250	MC4100 *surA*::*kan*	This study
JMR352	MC4100 *degP*::Tn*10*	This study
JMR354	JMR352 *surA*::*kan* (Ts)	This study
JMR580	JMR250/pAER1	This study
JMR582	JMR580 *degP*::Tn*10*	This study
JMR595	JMR582 *ara74*::*cam*	This study
CLC269	*degPS236A*	4
AR443	CLC269 *ara*$^+$	This study
AR444	AR443 Δ*skp zae-502*::Tn*10*	This study
AR445	AR444/pAER1	This study
AR446	AR445 *surA*::*kan*	This study

bility, we set out to test various combinations of null mutations in the genes that encode proteins thought to be involved in folding and/or targeting for synthetic phenotypes. Here we describe synthetic relationships between null mutations in the suspected chaperone genes *skp* and *surA*, as well as *surA* and *degP*. We propose that the synthetic lethality reflects redundant functions in the periplasm.

MATERIALS AND METHODS

Media and reagents. Media were prepared as described by Silhavy et al. (28). Antibiotics were used at the following concentrations in rich media: ampicillin, 125 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 25 μg/ml. Experiments with pAER1 were performed by using 0.2% (wt/vol) arabinose and in the presence of ampicillin (for plasmid maintenance). All of the restriction enzymes and T4 DNA ligase were used as directed by the manufacturer (New England Biolabs, Beverly, Mass.).

Bacterial strains and microbiological techniques. The bacterial strains used in this study are listed in Table 1. All of the strains are derivatives of *E. coli* K-12 strain MC4100. *surA*::*kan degP*::Tn*10* double mutants were constructed in two different ways. First, *surA*::*kan* was transduced into JMR352 (MC4100 *degP*::Tn*10*) at the permissive temperature (23°C), creating strain JMR354 (Table 1). Alternatively, JMR250 (MC4100 *surA*::*kan*) was transduced with *degP*::Tn10 in the presence of plasmid pAER1 and arabinose. Δ*skp surA*::*kan* (AR412) was constructed by transducing AR394 (MC4100 Δ*skp*/pAER1) with surA::*kan* in the presence of arabinose. Strains carrying plasmid pAER1 are either Ara^r (JMR595) or Ara$^+$ (AR412). *surA*::*kan*, *degP*::Tn*10*, and Δ*skp* alleles were gifts from R. Kolter, C. Georgopoulos, and U. Henning, respectively. The standard microbiological methods used for P1 transduction and transformation have been described previously (28).

Plasmid construction. Plasmid pAER1 was constructed as follows. High-copy-number pBAD18 (11)-based vector pBAD18-*surA* contains at its *Eco*RI site a 1,508-bp fragment of *surA* that includes the coding region and 187 and 34 bp of its 5′ and 3′ flanking regions, respectively (Martin Braun, unpublished data). On this plasmid, *surA* is under the control of the arabinose-inducible P_{BAD} promoter (11). To place *surA* on a low-copy-number vector, this plasmid was digested with *Cla*I and *Hin*dIII restriction enzymes, which yielded a 2,857-bp fragment carrying *araC*, P_{BAD}, and *surA*. Low-copy-number cloning vector pACYC177 (New England Biolabs) was digested with *Cla*I and *Hin*dIII, and a 3,512-bp fragment was purified and ligated to the entire *araC*-P_{BAD}-*surA* fragment, which disrupted the kanamycin resistance of pACYC177. The resulting construct (pAER1) was a low-copy-number, ampicillin-resistant plasmid that carried *surA* under the control of the arabinose-inducible P_{BAD} promoter.

Growth measurements. Δ*skp zae-502*::Tn*10* (AR299) and *degP*::Tn*10* (JMR352) strains carrying pAER1 were transduced with *surA*::*kan* in the presence of arabinose. Cells were grown overnight in Luria-Bertani (LB) media containing 0.2% arabinose and the appropriate antibiotics. Saturated cultures were washed by centrifugation twice with LB media lacking arabinose and were then subcultured at a dilution of 1:500 in LB media with or without arabinose. Growth was monitored by observing the optical density at 600 nm (OD_{600}) over time. After approximately five cell generations, cells were subcultured again at a dilution of 1:50 in fresh LB media with the appropriate antibiotics and in the presence or absence of arabinose. Growth was monitored until growth arrest occurred in the absence of arabinose. Growth experiments were performed at 37°C, unless otherwise noted. The M63 media used for growth experiments in minimal media were supplemented with 0.2% (wt/vol) maltose.

To assess the number of CFU, 10-μl aliquots were removed at various times, diluted with fresh LB media, and plated onto solid LB media containing antibiotics and 0.2% arabinose. After overnight growth at 37°C the numbers of CFU were determined.

Western blot analysis. One-milliliter samples of cells growing at 37°C were harvested by centrifugation. To ensure that the amounts of proteins were equal, cells were resuspended in a volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (15) determined by dividing the OD_{600} by three (which normalized the number of cells per milliliter for all samples). Cells were lysed by boiling the preparations for 10 min, and 10-μl samples were electrophoresed as described previously (15) on a sodium dodecyl sulfate–9% polyacrylamide gel, transferred to nitrocellulose membranes, and subjected to a Western blot analysis. Maltose binding protein (MBP) and LamB antisera were obtained from our laboratory stock (21) and were used at 1:3,000 dilutions. Enhanced chemiluminescence Western blotting reagents were purchased from Amersham Life Science, Piscataway, N.J.

Microscopy. Microscopic analysis was carried out with an Axiophot microscope with a 1.4 NA 100X Neofluor lens (Carl Zeiss, Inc.) or a 1.3 NA 100X UplanF1 iris lens (Olympus Corp.). Images were recorded with an SIT 3200 video camera equipped with a C2400 camera controller (Hamamatsu Corp.). Initially, images were processed with an Omnex image-processing unit (Imagen) and captured to a computer disk by using a Scion image capture board. Adobe Photoshop 5.5 was used to optimize contrast.

RESULTS

Δ*skp* and *surA*::*kan* produce a synthetic phenotype. The proposed roles of Skp and SurA as periplasmic chaperones (1–3, 8) led us to hypothesize that these two proteins are functionally redundant. To address this possibility, we attempted to construct a Δ*skp surA*::*kan* double null strain, but mutations in both *skp* and *surA* could not be tolerated in the same cell. Similar observations have been described by Behrens et al. (1). The lethality was alleviated by providing a wild-type copy of *surA* using plasmid pAER1. Growth of this strain, Δ*skp surA*::*kan*/pAER1, in LB media is arabinose dependent.

To more directly demonstrate synthetic lethality, Δ*skp surA*::*kan*/pAER1 was grown under permissive conditions (media containing arabinose) and then shifted to nonpermissive conditions (media lacking arabinose). Approximately 4.5 h after the transfer into nonpermissive media, growth of the Δ*skp surA*::*kan*/pAER1 mutant leveled off, while growth of the same mutant in the presence of arabinose continued (Fig. 1). The time of cessation of cell growth in the absence of arabinose corresponded to approximately 10 cell generations. Thus, the amount of SurA produced from plasmid pAER1 must be diluted approximately 1,000-fold before synthetic lethality is observed.

Δ*skp* and *surA*::*kan* are bacteriostatic under nonpermissive conditions. The growth of the Δ*skp surA*::*kan*/pAER1 mutant could cease under nonpermissive conditions either because cells in the population have stopped growing or because cells in the population are dying; that is to say, the effect of a Δ*skp surA*::*kan* double mutation might be either bacteriostatic or bactericidal. To distinguish between these two possibilities, we

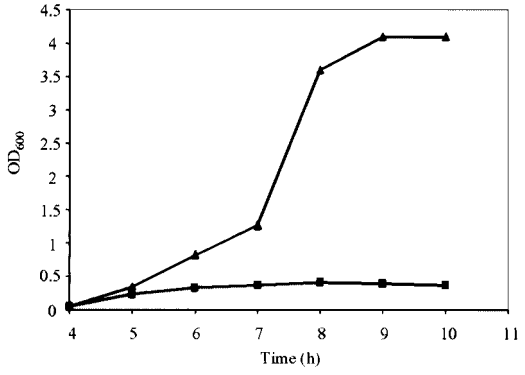

FIG. 1. Growth of Δ*skp surA*::*kan*/pAER1 requires arabinose. In the presence of arabinose (▲), cells produced a normal growth curve, while in the absence of arabinose (■), growth ceased after approximately 10 generations. The results of a representative experiment are shown. The 10-generation period was highly reproducible; 4 h corresponds to 4 h after the initial transfer to permissive or nonpermissive conditions.

determined the numbers of CFU produced over time by Δ*skp surA*::*kan*/pAER1 cells grown under both permissive and nonpermissive conditions (Fig. 2). The number of CFU increased steadily over time when Δ*skp surA*::*kan* double mutants were maintained in the presence of arabinose. In the absence of arabinose, the number of CFU remained relatively constant, indicating that the effect was bacteriostatic.

Envelope protein levels are reduced in a Δ*skp surA*::*kan* double mutant. Because both Skp and SurA have been implicated in the folding of envelope proteins, the levels of LamB, MBP, and OmpA were monitored in Δ*skp surA*::*kan*/pAER1 mutants over time after they were subcultured under nonpermissive conditions. Easily detectable levels of all three proteins were produced when the organisms were maintained in the presence of arabinose (Fig. 3). However, in cells subcultured without arabinose the levels of LamB, MBP, and OmpA were dramatically reduced approximately 6 h after subculturing. We were not able to determine from this experiment if the reduction in protein levels was due to a specific effect of the Δ*skp surA*::*kan* mutations that led to rapid degradation, to a more general downregulation of protein synthesis because of the extreme stress caused by the synthetic mutations, or to a combination of both effects. However, it is apparent from the cross-reacting bands shown in Fig. 3 that some proteins were not affected. The one cytoplasmic protein that we checked, RecA, was not affected either (data not shown). It could be that the levels of only envelope proteins are reduced, but more work is required to test this possibility.

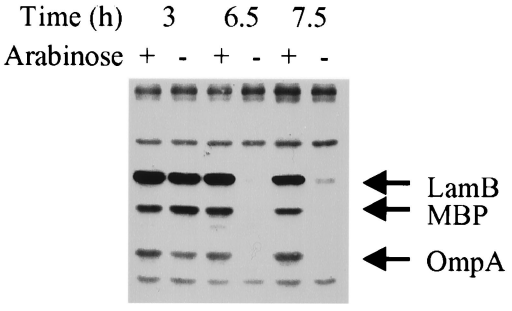

FIG. 3. Levels of envelope proteins are reduced in a Δ*skp surA*::*kan*/pAER1 mutant in the absence of arabinose. Western blots revealed that approximately 6.5 h after transfer into nonpermissive media the levels of LamB, MBP, and OmpA decreased dramatically.

Cellular morphology is altered in Δ*skp surA*::*kan* mutants. We examined the cellular morphology of Δ*skp surA*::*kan*/pAER1 cells grown under permissive and nonpermissive conditions (Fig. 4). As expected, in arabinose-containing media the mutants maintained the rod-shaped morphology of exponentially growing wild-type *E. coli* (Fig. 4A and C). In contrast, mutants grown in the absence of arabinose developed filaments that were approximately four cell lengths long as the growth arrest shown in Fig. 1 began to occur (Fig. 4B and D). The filaments may have resulted from a direct or indirect effect of a Δ*skp surA*::*kan* double mutation. For example, Skp and SurA may be intimately involved in the folding of cell division factors. Alternatively, filamentation may result from a cell division checkpoint that senses defects in envelope proteins and halts

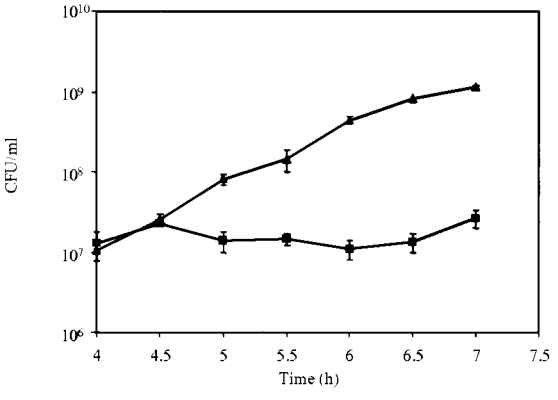

FIG. 2. Growth arrest in the absence of arabinose is bacteriostatic. The number of CFU increased over time when Δ*skp surA*::*kan*/pAER1 cells were maintained in the presence of arabinose (▲). In the absence of arabinose (■), the number of CFU remained relatively constant over time. The data are data from the experiment whose results are shown in Fig. 1.

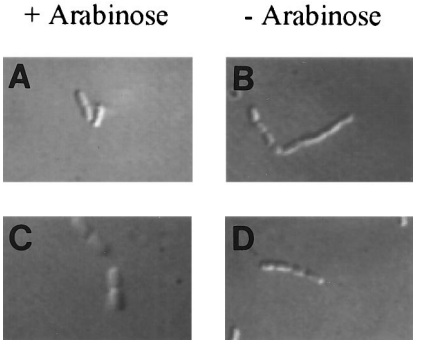

FIG. 4. Δ*skp surA*::*kan*/pAER1 mutants form filaments under nonpermissive conditions. Δ*skp surA*::*kan* cells formed filaments approximately 4 h after transfer to nonpermissive conditions, as growth arrest began to occur (B and D). These mutants maintained a rod-shaped morphology when they were complemented with pAER1 and grown in the presence of arabinose (A and C). Two random fields from each sample are shown.

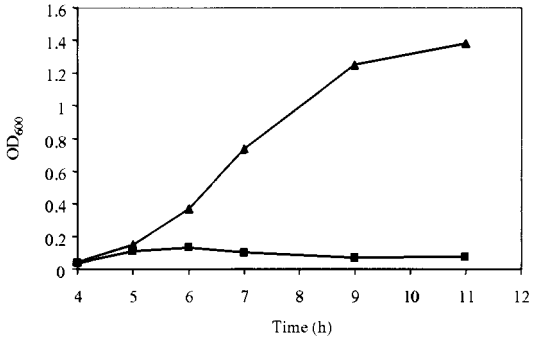

FIG. 5. Growth of *degP*::Tn*10 surA*::*kan*/pAER1 at 37°C requires arabinose. In the presence of arabinose (▲), cells produced a normal growth curve, while in the absence of arabinose (■), growth ceased after approximately 10 cell generations. The results of a typical experiment are shown.

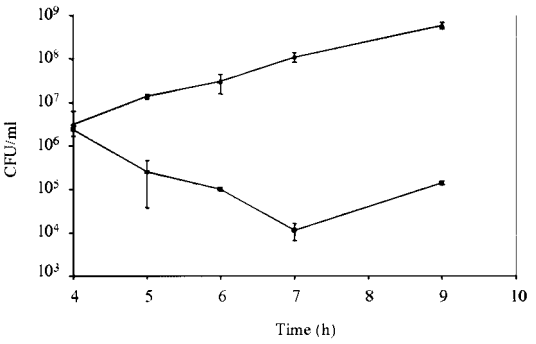

FIG. 6. Effect of *degP*::Tn*10 surA*::*kan* double mutations is bactericidal. The number of CFU increased over time when *degP*::Tn*10 surA*::*kan*/pAER1 cells were grown in the presence of arabinose (▲). In the absence of arabinose (■), the number of CFU decreased over time. The data are data from the experiment whose results are shown in Fig. 5.

cell division. We have not yet distinguished between these two possibilities.

***degP*::Tn*10* and *surA*::*kan* exhibit a synthetic phenotype.** The hypothesis that DegP plays a role as a periplasmic chaperone in addition to its role as a periplasmic protease suggested that this protein and SurA might also be functionally redundant. It has been shown that *degP*::Tn*10* mutants display a temperature-sensitive phenotype at temperatures above 37°C (18). *degP*::Tn*10 surA*::*kan* double mutants, however, grew only at 23°C and lower temperatures. In addition to this extreme temperature sensitivity, *degP*::Tn*10 surA*::*kan* double mutant strains exhibited increased sensitivity to certain antibiotics compared to *surA*::*kan* or *degP*::Tn*10* strains. The double mutant was up to fourfold more sensitive to novobiocin, amikacin, bacitracin, and vancomycin, as measured by disk sensitivity assays (data not shown).

The synthetic phenotype of a *degP*::Tn*10 surA*::*kan* double mutant was more directly characterized by growing cells under permissive conditions and then shifting them to nonpermissive conditions. These experiments were performed in two different ways. First, cells were grown at 23°C (the permissive temperature) and then shifted to 37°C (the nonpermissive temperature) (data not shown).

Alternatively, growth of the *degP*::Tn*10 surA*::*kan* double mutant at the nonpermissive temperature was restored in the presence of plasmid pAER1 by addition of arabinose to the media. *degP*::Tn*10 surA*::*kan* cells carrying pAER1 were grown at 37°C in media containing arabinose and then shifted to media lacking arabinose. Approximately 5 h after the shift to nonpermissive conditions, growth of the *degP*::Tn*10 surA*::*kan* mutant ceased, while growth of the same strain under permissive conditions continued (Fig. 5). Interestingly, the OD_{600} of this mutant actually decreased in the absence of arabinose, indicating that cell death occurred. The time that the growth stopped corresponded to approximately 10 cell generations, which was similar to the results obtained with Δ*skp surA*::*kan* double mutants.

The *degP*::Tn10 *surA*::*kan* synthetic phenotype reflects loss of chaperone activity. As noted above, the DegP protein is multifunctional, exhibiting both chaperone and protease activities (29). To examine which of these activities is important for the synthetic phenotypes observed with *surA*::*kan*, we employed plasmid pCLC1. This plasmid expresses mutant DegP (DegPS236A) that lacks protease activity yet presumably is still capable of chaperone activity (4). Strains transformed with pCLC1, as well as a control plasmid, were grown on LB medium plates at 23, 30, 37, and 42°C. Introduction of the protease-deficient *degP* gene allowed growth at 37°C and lower temperatures (data not shown). Similar results were obtained when a single copy of the protease-deficient *degP* gene was present on the chromosome. Therefore, the synthetic phenotype that was observed for *degP*::Tn*10 surA*::*kan* (lack of growth at temperatures above 23°C) could not be attributed to the loss of DegP protease activity.

***degP*::Tn*10 surA*::*kan* mutants are bactericidal under nonpermissive conditions.** To assess viability, we determined the number of CFU produced by *degP*::Tn*10 surA*::*kan*/pAER1 mutants in the presence or absence of arabinose (Fig. 6). As expected, the number of CFU steadily increased with time when the cells were grown in the presence of arabinose. However, in the absence of arabinose, the number of CFU decreased up to 2 orders of magnitude in 3 h. This is in contrast to the relatively constant number of CFU observed with Δ*skp surA*::*kan* mutants. These results indicate that the *degP*::Tn*10 surA*::*kan* mutations in combination have a bactericidal effect.

Envelope protein levels are reduced in a *degP*::Tn*10 surA*::*kan* mutant. The levels of LamB, MBP, and OmpA were monitored under both permissive and nonpermissive conditions. While MBP, LamB, and OmpA were easily detected when the *degP*::Tn*10 surA*::*kan*/pAER1 strain was maintained in the presence of arabinose, their levels were dramatically reduced in the absence of arabinose. Western blot analysis gave results very similar to those shown in Fig. 3 for the Δ*skp surA*::*kan* mutant (data not shown).

Cellular morphology is not altered in *degP*::Tn*10 surA*::*kan* mutants. As described above, Δ*skp surA*::*kan*/pAER1 mutants undergo a striking morphological change under nonpermissive conditions: they form filaments in the absence of arabinose. However, *degP*::Tn*10 surA*::*kan*/pAER1 mutants exhibited no morphological changes. Both in the presence and in the absence of arabinose, these mutants appeared to have a normal rod shape (data not shown). The cell death observed with the

degP::Tn*10* *surA*::*kan* combination likely precluded morphological changes like those seen with Δ*skp surA*::*kan*, which is bacteriostatic.

Loss of DegP protease activity leads to cell death in Δ*skp surA*::*kan* mutants. We assume that it is the accumulation of unfolded proteins in the periplasm that causes the synthetic phenotypes observed with Δ*skp surA*::*kan* and *degP*::Tn*10* *surA*::*kan* mutants. Yet why is the former bacteriostatic and the latter bactericidal? We suggest that cell death is caused by a lack of the DegP protease.

The DegPS236A mutant protein lacks the protease function (4) but retains chaperone activity. Indeed, it can complement the synthetic phenotype of the *degP*::Tn*10* *surA*::*kan* double mutant (see above). We constructed a strain carrying *degPS236A* on the chromosome, in addition to Δ*skp*, *surA*::*kan*, and pAER1. We monitored the growth of this mutant, *degPS236A* Δ*skp surA*::*kan*/pAER1, in both the presence and the absence of arabinose. Similar to the results obtained with a *degP*::Tn*10* *surA*::*kan*/pAER1 mutant, growth of *degPS236A* Δ*skp surA*::*kan*/pAER1 ceased after approximately 10 cell generations (data not shown). However, the OD_{600} of this mutant actually decreased more than fivefold after it was maintained under nonpermissive conditions for approximately 5 h.

To test viability, we looked at the number of CFU produced by *degPS236A* Δ*skp surA*::*kan*/pAER1 along the growth curve under both permissive and nonpermissive conditions. As expected, in the presence of arabinose the number of CFU increased over time. Strikingly, in the absence of arabinose the number of CFU decreased approximately 1 log concurrent with the decrease in OD_{600} (data not shown).

In the presence of DegP protease, a Δ*skp surA*::*kan* double mutation is bacteriostatic. When we took away the DegP protease function, the Δ*skp*, *surA*::*kan* mutations became bactericidal. Together, these results demonstrate that DegP protease activity can combat the bactericidal consequences of compromised periplasmic chaperone activity.

Synthetic lethality of Δ*skp surA*::*kan* and *degP*::*Tn10 surA*::*kan* mutants is not observed in minimal media. Pulse-chase analysis was needed to distinguish whether the reduction in the levels of envelope proteins observed under nonpermissive conditions is due to synthesis defects or due to rapid degradation. To enable such biochemical studies of Δ*skp surA*::*kan*/pAER1 and *degP*::*Tn10* *surA*::*kan*/pAER1 double mutants, we attempted to characterize the synthetic phenotypes in minimal media. However, the two double mutants grew similarly under both permissive and nonpermissive conditions in maltose minimal media.

We monitored cultures of Δ*skp surA*::*kan*/pAER1 for approximately 32 cell generations and were not able to see a growth defect. To test for suppressors which may have overtaken the population, we subcultured organisms in rich media with or without arabinose at various times along the growth curve. As described above, cells subcultured in media without arabinose stopped growing after approximately 10 cell generations. In addition, at various times cells were plated on minimal agar either with or without arabinose. Colonies grew normally under both conditions. Thus, the growth defects caused by Δ*skp surA*::*kan* and *degP*::*Tn10* *surA*::*kan* do not occur in minimal media.

DISCUSSION

Allele-specific synthetic lethality has been used to demonstrate direct protein-protein interactions (10, 12). In contrast, the mutations used in this study are recessive null mutations, and thus, the genetic interactions are not allele specific. We can imagine three possible explanations for the synthetic phenotypes observed with certain pairwise combinations of *skp*, *degP*, and *surA* null mutations.

First, two mutations that affect growth for entirely different reasons might be lethal when they are combined, and this could be mistaken for synthetic lethality. We do not favor this explanation for our observations because the lethality observed with the pairwise combinations described here does not correlate with the sickness caused by the individual mutations. Specifically, the *degP* null allele confers the most drastic phenotype of the three mutations that we investigated. Strains carrying *degP*::Tn*10* show growth defects at temperatures greater than 37°C (18). The *surA*::*kan* mutation confers some defects, such as sensitivity to hydrophobic dyes and antibiotics, as well as mucoid colony formation, both of which are indicative of outer membrane defects (16, 17, 22, 25). In contrast, the Δ*skp* mutation confers no visible phenotype that indicates sickness. Yet despite the fact that Δ*skp* strains are not sick, they exhibit a lethal phenotype when this mutation is combined with *surA*::*kan* but not when it is combined with *degP*::Tn*10*. Although there have been reports of a synthetic conditional phenotype in Δ*skp degP*::Tn*10* mutants at temperatures greater than 37°C (26) or 39°C (6), we did not see this defect in our strain background. Even so, the restrictive temperature used in these experiments is only a few degrees lower than that of the *degP* single mutant (6, 18, 26). In contrast, the results presented here show that in the *degP::*Tn*10* *surA*::*kan* mutant the restrictive temperature is decreased almost 20°C.

A second possible explanation for the synthetic relationships reported here is that DegP, Skp, and SurA are all part of a larger complex. In this scenario, if one component of the complex is taken away, as it is in the single mutants, the complex remains functional. However, if two components of the complex are absent, the complex falls apart, resulting in a lethal phenotype. Such relationships have been reported for multiprotein complexes in *Saccharomyces cerevisiae* (14, 27). However, we do not favor this explanation for several reasons. First, the fact that *degP*::Tn*10* *surA*::*kan* phenotypes differ from Δ*skp surA*::*kan* phenotypes requires that SurA be a part of two different complexes. Moreover, SurA has been extensively studied biochemically, and no evidence for involvement in any complex has been reported.

The third and, we believe, the most likely explanation for the pattern of synthetic lethality reported here is that both Skp and DegP share a redundant function with SurA. The redundant function that we favor is periplasmic chaperone activity. Indeed, biochemical evidence for chaperone activity has been presented for all three proteins (1, 3, 29).

To account for the fact that only certain pairwise combinations of *skp*, *degP*, and *surA* null mutations cause synthetic lethality, we propose that there are two pathways in the periplasm for chaperone activity. DegP and Skp are in one pathway, and SurA is in a separate, parallel pathway. Thus, losing one component of either pathway alone is tolerated

because the other, parallel pathway can still function. However, losing one component of each pathway simultaneously results in a lethal phenotype because both pathways for chaperone activity have been compromised. Similar logic was used by Miller and Rose to propose parallel pathways for yeast nuclear migration (20).

The parallel pathway model accounts for the pattern of synthetic lethality reported here, but what about the phenotypic differences between a Δ*skp surA*::*kan* mutant and a *degP*::Tn*10 surA*::*kan* mutant? Mutations in *degP* and *surA* together have a bactericidal effect, while the Δ*skp surA*::*kan* combination is bacteriostatic. How could blocking parallel pathways in different ways have such different effects?

It has previously been shown that *degP* mutants are bactericidal on their own when they are grown at restrictive temperatures (18). We propose that in both the *degP*::Tn*10* single mutant (at 42°C) and the *degP*::Tn*10 surA*::*kan* double mutant there are unfolded proteins in the cell envelope (the former because of the high temperature and the latter because of loss of both chaperone pathways). These unfolded proteins accumulate and cause cell death because the protease activity of DegP is lost in both cases. It is important to note, however, that the synthetic lethality observed in *surA* and *degP* mutants is due to loss of chaperone activity alone as the phenotype can be complemented by *degP* lacking protease activity. The Δ*skp surA*::*kan* combination is not bactericidal because the DegP protease is still present to combat the accumulation of unfolded proteins that occurs as a consequence of losing both chaperone pathways. Indeed, we have shown that removing DegP protease activity leads to cell death in a Δ*skp surA*::*kan* double mutant. We believe that the relationship between cell death and the DegP protease strengthens the hypothesis that the synthetic phenotypes described here are due to loss of periplasmic chaperone activity.

Another major difference between the two synthetic relationships is the fact that Δ*skp surA*::*kan* mutants form filaments while *degP*::Tn*10 surA*::*kan* mutants do not. This is easily explained because *degP*::Tn*10 surA*::*kan* cells die and most likely do not form filaments because of this. Indeed, in the absence of DegP protease activity, Δ*skp surA*::*kan* mutants die before filamentation is observed.

Remarkably, the synthetic phenotypes which we observed do not occur in minimal media. Perhaps under these conditions growth is slowed enough that a crippled pathway is sufficient. Alternatively, there may be another chaperone pathway(s) that functions under such conditions. In our studies we used depletion, and it was almost 10 cell generations before lethality was observed. By this time, expression of envelope proteins in the stressed cells was reduced and a compensatory stress response(s) may have been induced.

We attempted to label the double mutant cells in rich media by using the protocol of Doerrler et al. (9). At the time when growth defects were observed under nonpermissive conditions (10 generations) labeling was very poor. Although not conclusive, the data suggest that synthesis of LamB and MBP is defective. However, even though several proteins exhibited similar labeling patterns under both permissive and nonpermissive conditions, we cannot rule out the possibility of a more general synthesis defect. Furthermore, our inability to reproduce the growth defect in minimal media and difficulty with labeling in rich media prevented us from determining conclusively whether the reduced envelope protein levels are the result of a synthesis defect or protein degradation or both. Unfortunately, such technical difficulties pose a serious problem for analysis of periplasmic chaperone activity in vivo. Temperature-sensitive mutations may provide a solution that will allow more informative biochemical analysis.

The data upon which the two-pathway model rests are genetic, and we do not know the biochemical basis for the functional separation. It could be that each pathway has certain preferred substrates. In any event, it should be possible to use genetics to classify other periplasmic folding factors with respect to the pathways. For example, there have been reports of synthetic lethality with *skp* and *fkpA* mutants (6). Such an interaction would place FkpA in the SurA pathway in our model of periplasmic chaperone activity.

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