JOURNAL OF BACTERIOLOGY, May 2006, p. 3494–3497 Vol. 188, No. 10
0021-9193/06/$08.00+0 doi:10.1128/JB.188.10.3494–3497.2006

Increased Persistence in *Escherichia coli* Caused by Controlled Expression of Toxins or Other Unrelated Proteins

Nora Vázquez-Laslop,* Hyunwoo Lee, and Alexander A. Neyfakh

Center for Pharmaceutical Biotechnology, University of Illinois, Chicago, Illinois 60607

Received 16 January 2006/Accepted 7 March 2006

Bacterial populations contain persisters, cells which survive exposure to bactericidal antibiotics and other lethal factors. Persisters do not have a genetic resistance mechanism, and their means to tolerate killing remain unknown. In exponentially growing populations of *Escherichia coli* the frequency of persister formation usually is 10^{-7} to 10^{-5}. It has been shown that cells overexpressing either of the toxic proteins HipA and RelE, both members of the bacterial toxin-antitoxin (TA) modules, have the ability to form more persisters, suggesting a specific role for these toxins in the mechanism of persistence. However, here we show that cells expressing proteins that are unrelated to TA modules but which become toxic when ectopically expressed, chaperone DnaJ and protein PmrC of *Salmonella enterica*, also form 100- to 1,000-fold more persisters. Thus, persistence is linked not only to toxicity caused by expression of HipA or dedicated toxins but also to expression of other unrelated proteins.

A small number of cells in a bacterial population can survive treatment with lethal concentrations of bactericidal antibiotics. These survivors, which appear with a frequency of $\sim 10^{-6}$, are known as persisters (3). The nature of their tolerance to killing and the molecular mechanisms triggering the persister state remain unknown.

Persisters do not have a genetic resistance mechanism, and their progeny are fully sensitive to antibiotics (3, 14, 18). The only gene that has been linked to persistence in *Escherichia coli* has been *hipA* (4, 5, 15, 16, 19, 20, 23). A mutation in this gene, the *hipA7* mutation, was identified because cells carrying this mutant gene formed persisters at a higher frequency (10^{-1} to 10^{-3}) compared to the wild-type strain (16, 19, 23). The high frequency of persister formation is not restricted to cells with the chromosomal hipA7 mutation; it has also been shown for cells overexpressing the toxic wild-type HipA (4, 7, 16, 20). Cells expressing this protein form 10- to 1,000-fold more persisters tolerant to bactericidal antibiotics with different mechanisms of actions, such as ampicillin and ciprofloxacin (7).

It has been suggested that the *hipA* gene, encoding the toxic factor, together with *hipB*, encoding a putative antidote, constitutes one of the several toxin-antitoxin (TA) modules present in bacteria (8, 9). Similar to typical TA operons, like *relBE* and *mazEF*, *hipB* and *hipA* are organized in an operon with the gene encoding the antitoxin, *hipB*, located upstream of *hipA*, the toxin gene (4, 5, 8, 9).

It has been shown recently that, similar to HipA, cells overproducing the toxin RelE, another member of a TA module, form 10- to 10,000-fold more persisters (15). Both proteins, HipA and RelE, have the effect of slowing down or even stopping cell division, raising the possibility that toxins from the TA modules have a role in increasing the fraction of dormant or nongrowing cells in a population, the fraction, it has been suggested, that constitute the persister cells (2, 3, 14, 15, 18). The various roles in cell physiology of toxins RelE and MazF are most likely related to their specific activity in inhibiting translation by cleaving mRNA at particular sites (6, 21). It is unclear, however, whether the increase in the number of persisters observed when HipA (7) or RelE (15) is overexpressed from plasmids is a result of a specific dedicated role of these toxins in the mechanism of forming persisters. It is known that expression of a wide variety of recombinant proteins, even those that are not dedicated toxins, often has a detrimental effect on the growth of bacteria (11). Evidently, most of these proteins share no homology in sequence, gene organization, or function with the toxins of the TA modules, and thus their effects on cell physiology are probably of a different nature.

In this work, we asked whether the increase in frequency of persister formation is specific to cells overexpressing toxins from the TA modules or if cells expressing proteins that become toxic when produced from plasmids would also show high persistence.

MATERIALS AND METHODS

Strains and plasmids. Plasmids $pBAD_{18CM}$ and $pBAD_{22CM}$ were used as cloning vectors for expression of *E. coli* genes *thrB*, *dnaJ*, *mazF*, and *hipA* and *Salmonella enterica* gene *pmrC*. $pBAD_{CM}$ plasmids were prepared by replacing the ampicillin resistance gene with the chloramphenicol resistance gene. Genes to be cloned were PCR amplified using pairs of primers (Table 1) and high-fidelity AccuPrime *Taq* DNA polymerase (Invitrogen). DNA from *E. coli* strain MG1655 was prepared using a genomic DNA isolation kit (Sigma; catalog no. NA2120) and used as a template for amplification of *hipA*, *mazF*, *dnaJ*, and *thrB*. p*pmrC* plasmid (17) carrying the *pmrC* gene of *Salmonella enterica* serovar Typhimurium 14028s was used as a template for PCR amplification of *pmrC*. The amplified PCR products were cut with the appropriate restriction enzymes and cloned in the corresponding sites of the vector plasmids (Table 1).

Escherichia coli strain LMG194 (F$^-$ Δ*lacX74 galE thi rpsL* Δ*phoA* (PvuII) Δ*ara714 leu*::Tn*10*) was used as the host strain for the empty vector $pBAD_{22CM}$ and all the constructs described in Table 1.

Determination of the effect of protein overexpression on the different strains. LMG194 cells carrying $pBAD_{CM}$ plasmids were streaked on Luria-Bertani (LB) agar plates containing 0.2% glucose and 25 μg/ml chloramphenicol. Overnight cultures were grown from single colonies in RMG minimal medium (2%

* Corresponding author. Mailing address: Center for Pharmaceutical Biotechnology, m/c 870, University of Illinois, 900 S. Ashland Ave., Chicago, IL 60607. Phone: (312) 996-3371. Fax: (312) 413-4034. E-mail: nvazquez@uic.edu.

TABLE 1. Plasmids and primers used in this study

Plasmid	Primers[a]	Source of template DNA[b]	Relevant features
$pBAD_{22}$*thrB*	5′-TTAGGAATTCGACATGGTTAAAGTTTATGCCC, 5′-CCTGAAGCTTGTGATCTTTCAGATTGTAGAGT	MG1655	*thrB* gene in EcoRI-HindIII sites of $pBAD_{22}$
$pBAD_{22}$*dnaJ*	5′-CAATGAATTCAAGATGGCTAAGCAAGATTATTA, 5′-GCAGTCTAGAGGGGAGGTTAGCGGGTCAG	MG1655	*dnaJ* gene in EcoRI-XbaI sites of $pBAD_{22}$
$pBAD_{18}$*pmrC*	5′-TGGAGAATTCAACATGTTAAAGCGCTT, 5′-TCAGAAGCTTCATTCGCTTAGTCTCCT	p*pmrC* (17)	*pmrC* gene in EcoRI-HindIII sites of $pBAD_{22}$
$pBAD_{22}$*mazF*	5′-AGGAGAATTCGTAATGGTAAGCCGATACGTA, 5′-ATAGAAGCTTGTTAGTAACACTACCCAATCAG	MG1655	*mazF* gene in EcoRI-HindIII sites of $pBAD_{22}$
$pBAD_{CM}$*hipA*	5′-GTGGGCATGCCTAAACTTGTCACTTGG, 5′-ACCCGAATTCTCAC**TTA**CTACCGTATTCTCG	MG1655	*hipA* gene in NcoI-EcoRI sites of pBADMycHis; a stop codon (boldface) was introduced to avoid expression of Myc epitope and His tail

[a] Primers used for PCR amplification of the genes cloned in the corresponding plasmids. Restriction sites are underlined.
[b] Template DNA used for PCR amplification of the genes.

Casamino Acids, 1× M9 salts, 1 mM $MgCl_2$, and 1% glycerol) supplemented with 25 μg/ml chloramphenicol (RMG-CM). Aliquots of 0.85 ml were mixed with 0.15 ml of sterile glycerol, snap-frozen in a dry ice-ethanol bath, and kept at −80°C.

For the protein expression experiments, cultures were started from these glycerol stocks. After an overnight growth in RMG-CM medium, cultures were diluted 100-fold into 30 ml of fresh medium in 125-ml Erlenmeyer flasks. Flasks were shaken at 160 rpm in a platform shaker (Innova 2300; New Brunswick Scientific) at 37°C. When cell densities of the cultures reached an optical density at 600 nm (OD_{600}) of ~0.15, 3- to 5-ml aliquots were transferred to 30- by 115-mm conical plastic tubes. Arabinose was added at different concentrations for an adequate time period (see below). Tubes were placed at an inclination of 45°, and shaking continued under the same conditions. Cell growth was determined by monitoring the OD_{600}. The numbers of viable cells (CFU) in the cultures, before and after the addition of arabinose, were determined as follows. Ten-microliter aliquots were serially diluted in a 10-fold fashion. One hundred-microliter aliquots from these dilutions were plated on LB agar-chloramphenicol plates and kept overnight at 37°C. Due to the toxicity of the proteins expressed, a drop in the cell counts was expected. Therefore, it was important to carry out a wide range of dilutions in order to use the adequate ones to reliably determine CFU.

Determination of persistence frequency in cells overexpressing proteins. To determine the number of persisters formed by cells expressing proteins from pBAD vectors, cells were grown and protein expression was induced as described above. We found that keeping these conditions was critical to obtain reproducible persistence frequencies between experiments. Similar observations about different factors influencing reproducibility of the persistence frequency have been reported previously (15). An antibiotic, ampicillin (Fisher Scientific BP1760) or ciprofloxacin (Bayer Co.), was added from stock solutions to a final concentration of 100 μg/ml or 0.4 μg/ml, respectively. Incubation continued for 4 h. Chloramphenicol (25 μg/ml) was kept throughout the experiments to ensure the stability of the plasmids. In order to determine viable-cell counts in the cultures after incubation with the antibiotics, 1-ml aliquots were removed and cells were spun and resuspended in fresh medium without antibiotics. Determination of viable cells was performed as described above. Persistence frequency was calculated by dividing the number of CFU/ml in the culture after incubation with the antibiotic by the number of CFU/ml in the culture before adding the antibiotic.

RESULTS AND DISCUSSION

Cells overexpressing toxins from TA modules show an increase in persistence frequency (4, 7, 15, 16, 20). Here we asked if expression of proteins that become toxic when ectopically produced also results in a higher frequency of persister formation. In order to test this, we chose to express in *E. coli* cells, in parallel to the toxins from the TA modules HipA and MazF, two unrelated proteins. One was protein DnaJ from the chaperone system DnaJ/DnaK/GrpE (12), which becomes toxic when overexpressed from a high-copy-number plasmid (http://ecoli.aist-nara.ac.jp/gb5/Resources/archive/archive.html). The second protein, PmrC from *Salmonella enterica* serovar Typhimurium 14028s, an enzyme that transfers phosphoethanolamine to lipid A, is also toxic to *E. coli* cells when expressed from a plasmid (17). In addition, ectopic expression of *thrB* was used as a control, since threonine B, a component of homoserine kinase, can be produced to high levels with no detrimental consequences for cell growth (13).

Due to the expected differential toxicity of the proteins, it was important to express them in a tightly regulated system. The PCR-amplified genes *hipA*, *mazF*, and *dnaJ* (from *E. coli* K-12 strain MG1655) and *pmrC* (from the p*pmrC* plasmid described in reference 17) were cloned under the control of the P_{araBAD} promoter into the $pBAD_{CM}$ vector, carrying a chloramphenicol resistance determinant (see above). *E. coli* LMG194 cells unable to metabolize L-arabinose (10) were transformed with the obtained expression plasmids and were grown in RM minimal medium supplemented with 1% glycerol and chloramphenicol (25 μg/ml). In this expression system, the activity of the P_{BAD} promoter could be tightly controlled by the concentration of arabinose, and the basal levels of expression of the cloned genes are negligible (10).

To test how expression of the cloned genes affects cell growth, arabinose at a final concentration of 0.2% was added to logarithmically growing cultures at 37°C. Overexpression of the control protein ThrB slowed down the growth rate of the cells in a relatively mild fashion. In contrast, expression of toxins HipA and MazF, as well as proteins DnaJ and PmrC, caused a complete growth arrest after some time at this saturating concentration of inducer (Fig. 1A). The toxic effect of the toxins HipA and MazF or the proteins DnaJ and PmrC on dividing cells could be reached at different levels of induction and, generally, could be bacteriostatic or bactericidal. This fact makes difficult the analysis and interpretation of any experiments comparing cells producing these proteins. In the case of TA module proteins, it has been reported that ectopic overexpression of MazF may have a lethal effect depending on the conditions and duration of the toxin expression (1, 22). Similarly, overproduction of HipA has been shown to cause occasional lysis of the cells (16). No information in this regard was available for the effect of overexpressing DnaJ or PmrC. Therefore, it was important to adjust the expression of the

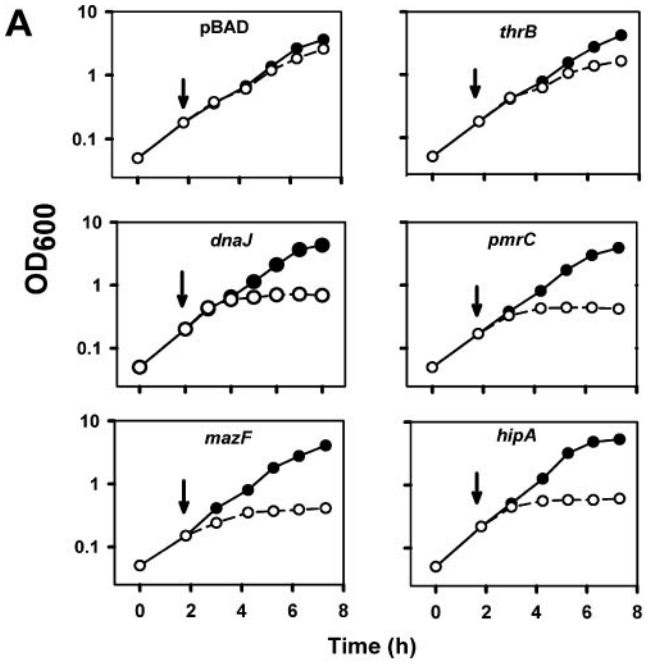

FIG. 1. Effect of toxic proteins on the growth and viability of *E. coli* cells. A. Growth curves of LMG194 cells transformed with pBAD plasmids to induce expression of the indicated genes. Arrows show the moment of addition of arabinose. Solid circles, no arabinose; open circles, arabinose added. B. Number of live cells in strains producing toxic proteins after optimizing the concentration of inducer (see text). Arabinose concentrations were as follows: 0.2% for the empty vector and *thrB*; 0.05% for *dnaJ*; and 0.015% for *pmrC*, *mazF*, and *hipA*. White bars, no arabinose; gray bars, arabinose added.

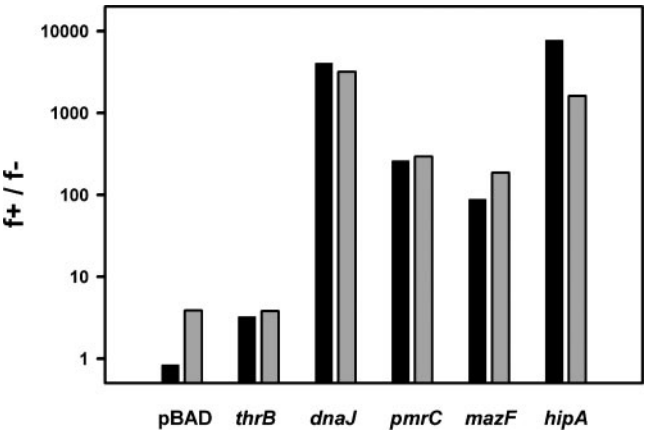

FIG. 2. Frequency of persister formation in strains expressing toxic proteins. Cells producing proteins under the conditions indicated in Fig. 1B were challenged with ampicillin (black bars) or ciprofloxacin (gray bars). The persistence frequency (f^+) was estimated relative to that for cells challenged with the antibiotics but grown without arabinose (f^-). A value of 1 would indicate no effect of protein overexpression on the frequency of persister formation.

different proteins so that they would produce comparably mild inhibitory effects on the growth rate of the cells. In order to achieve this, logarithmically growing cultures of each strain were induced with different concentrations of arabinose. The densities (OD_{600}) and numbers of viable cells (CFU) of the cultures were determined over time (not shown). Based on these data, induction conditions were chosen under which expression of the cloned proteins caused a minimal but evident decrease in the culture densities compared to the uninduced controls. Under these conditions, protein expression barely affected (strain with empty vector and strains expressing ThrB, PmrC, and MazF) or decreased only moderately (strains expressing DnaJ and HipA) the number of viable cells in the cultures (Fig. 1B). The level of protein expression in these cultures was examined by gel electrophoresis (not shown). A 34-kDa band corresponding to ThrB could be clearly identified, in agreement with the fact that ThrB can reach high expression levels without having any important effect on the growth rate or the number of cells (Fig. 1). In contrast, no bands could be detected for either DnaJ, PmrC, MazF, or HipA on Coomassie-stained gels. This suggests that the toxic effect of all these proteins is attained at low levels of induction. It should be noted that at low level of induction protein expression may vary between individual cells (24).

The frequency of persister formation in cells expressing proteins under the conditions described above was determined. In order to do this, bactericidal antibiotics with unrelated mechanisms of action, ampicillin and ciprofloxacin, were used. Logarithmically growing cells were induced with adequate concentrations of arabinose for 1 (strains expressing ThrB, HipA, MazF, or PmrC or carrying an empty vector) or 2 h (DnaJ-producing strain). The induced cultures were then challenged with either 0.4 μg/ml of ciprofloxacin or 100 μg/ml of ampicillin for 4 h. In order to determine persistence frequency in the induced cultures, a fraction of the cells before and after the antibiotic treatment were washed with fresh medium and plated on LB agar-chloramphenicol plates containing 0.2% glucose to prevent any further production of the toxins. The frequency of persister formation was also determined for each strain grown in the absence of inducer. Figure 2 shows the ratios of the frequency of appearance of persisters in strains overproducing the cloned protein compared to the frequency in the absence of the inducer. Overexpression of the control protein ThrB, which showed only a mild negative effect on the growth of the cells, did not significantly increase the frequency of persisters. In contrast, strains expressing at limited induction levels members of the TA pairs MazF and HipA increased the frequency of formation of both ampicillin and ciprofloxacin persisters 100- to 10,000-fold. Remarkably, a similar increment

of persistence was also detected for the strains expressing the chaperone DnaJ or even the foreign protein PmrC (100- to 1,000-fold), under comparably low levels of induction. These results show that, at least in the mid-log-phase cells used for these experiments, the increase in number of persisters is not unique to strains expressing HipA or other dedicated toxins. Instead, cells expressing proteins totally unrelated to the TA modules, which become toxic when ectopically expressed, also have the ability to form more persisters. It has been suggested that the formation of persisters during stationary phase is highly dependent on the *hipBA* locus, since its deletion causes a sharp drop in persister formation during this stage (15). However, to the best of our knowledge, no systematic effort to study the effect of other gene deletions on persistence of stationary-phase cells has been reported. Therefore, it is possible that deletions of genes other than *hipA* might also have the effect of diminishing persistence frequency.

One of the main questions about the phenomenon of persistence is whether bacterial cells have evolved a dedicated mechanism that allows them to survive lethal stresses including exposure to bactericidal antibiotics. Such a mechanism may potentially be based on a deliberate or sporadic expression of toxin proteins from the TA modules in a small fraction of cells to maintain them in a dormant or nongrowing stress-tolerant state (9, 15, 18). However, our findings suggest that limited levels of induction of unrelated proteins also increase persistence. Deleterious effects of such proteins will be attained at different intracellular concentrations, and the mechanism of their toxicity will likely have nonspecific targets. Given the low frequency of persisters in a wild-type population ($\sim 10^{-6}$), it is conceivable that stochastic variations or illicit gene expression would lead to rare toxic levels of a broad variety of proteins in a small fraction of cells, increasing their chances to become persisters.

ACKNOWLEDGMENTS

We are grateful to G. H. Araia for excellent technical assistance. The critical reading of the manuscript by A. S. Mankin is greatly appreciated.

This work was supported by Public Health Service grants AI-049214 and AI- 056575 from the National Institute of Allergy and Infectious Diseases to A.N.

REFERENCES

1. **Amitai, S., Y. Yassin, and H. Engelberg-Kulka.** 2004. MazF-mediated cell death in *Escherichia coli*: a point of no return. J. Bacteriol. **186:**8295–8300.
2. **Balaban, N. Q., J. Merrin, R. Chait, L. Kowalik, and S. Lieber.** 2004. Bacterial persistence as a phenotypic switch. Science **305:**1622–1625.
3. **Bigger, J. W.** 1944. Treatment of staphyloccocal infections with penicillin. Lancet **ii:**497–500.
4. **Black, D. S., A. J. Kelly, M. J. Mardis, and H. S. Moyed.** 1991. Structure and organization of *hip*, an operon that effects lethality due to inhibition of peptidoglycan or DNA synthesis. J. Bacteriol. **173:**5732–5739.
5. **Black, D. S., B. Irwin, and H. S. Moyed.** 1994. Autoregulation of *hip*, and operon that affects lethality due to inhibition of peptidoglycan or DNA synthesis. J. Bacteriol. **176:**4081–4091.
6. **Christensen, S. K., K. Pedersen, F. G. Hansen, and K. Gerdes.** 2003. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. J. Mol. Biol. **332:**809–819.
7. **Falla, T. J., and I. Chopra.** 1998. Joint tolerance to β-lactam and fluoroquinolone antibiotics in *Escherichia coli* results from overexpression of *hipA*. Antimicrob. Agents Chemother. **42:**3282–3284.
8. **Falla, T. J., and I. Chopra.** 1999. Stabilization of *Rhizobium* symbiosis plasmids. Microbiology **145:**515–516.
9. **Gerdes, K., S. K. Christensen, and A. Løbner-Olesen.** 2005. Prokaryotic toxin-antitoxin stress loci. Nat. Rev. Microbiol. **3:**371–382.
10. **Guzman, L.-M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:**4121–4130.
11. **Hoffmann, F., and U. Rinas.** 2004. Stress induced by recombinant protein production in *Escherichia coli*. Adv. Biochem. Eng. Biotechnol. **89:**73–92.
12. **Houry, W. A.** 2001. Chaperone-assisted protein folding in the cell cytoplasm. Curr. Protein Pept. Sci. **2:**227–244.
13. **Huo, X., and R. E. Viola.** 1996. Functional group characterization of homoserine kinase from *Escherichia coli*. Arch. Biochem. Biophys. **330:**373–379.
14. **Keren, I., N. Kaldalu, A. Spoering, Y. Wang, and K. Lewis.** 2004. Persister cells and tolerance to antimicrobials. FEMS Microbiol. Lett. **230:**13–18.
15. **Keren, I., D. Shah, A. Spoering, N. Kaldalu, and K. Lewis.** 2004. Specialized persister cells and mechanism of multidrug tolerance in *Escherichia coli*. J. Bacteriol. **186:**8172–8180.
16. **Korch, S. B., T. A. Henderson, and T. M. Hill.** 2003. Characterization of the *hipA7* allele of *Escherichia coli* and evidence that high persistence is governed by (p)ppGpp synthesis. Mol. Microbiol. **50:**1199–1213.
17. **Lee, H., F.-F. Hsu, J. Turk, and E. A. Groisman.** 2004. The PmrA-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. J. Bacteriol. **186:**4124–4133.
18. **Lewis, K.** 2000. Programmed death in bacteria. Microbiol. Mol. Biol. Rev. **64:**503–514.
19. **Moyed, H. S., and K. P. Bertrand.** 1983. *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. J. Bacteriol. **155:**768–775.
20. **Moyed, H. S., and S. H. Broderick.** 1986. Molecular cloning and expression of *hipA*, a gene of *Escherichia coli* that affects frequency of persistence after inhibition of murein synthesis. J. Bacteriol. **166:**399–403.
21. **Pedersen, K., A. V. Zavialov, M. Y. Pavlov, J. Elf, K. Gerdes, and M. Ehrenberg.** 2003. The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. Cell **112:**131–140.
22. **Pedersen, K., S. K. Christensen, and K. Gerdes.** 2002. Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. Mol. Microbiol. **45:**501–510.
23. **Scherrer, R., and H. S. Moyed.** 1988. Conditional impairment of cell division and altered lethality in *hipA* mutants of *Escherichia coli* K-12. J. Bacteriol. **170:**3321–3326.
24. **Siegele, D. A., and J. C. Hu.** 1997. Gene expression from plasmids containing the *araBAD* promoter at subsaturating inducer concentrations represents mixed populations. Proc. Natl. Acad. Sci. USA **94:**8168–8172.