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Genetics

Addiction protein Phd of plasmid prophage P1 is a substrate of the ClpXP serine protease of *Escherichia coli*

(bacteriophage P1/plasmid stability/post-segregational killing/ATP-dependent protease/homeostasis)

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ABSTRACT **Plasmid-encoded addiction genes augment the apparent stability of various low copy number bacterial plasmids by selectively killing plasmid-free (cured) segregants or their progeny. The addiction module of plasmid prophage P1 consists of a pair of genes called *phd* and *doc*. Phd serves to *p*revent *h*ost *d*eath when the prophage is retained and, should retention mechanisms fail, Doc causes *d*eath *o*n *c*uring. Doc acts as a cell toxin to which Phd is an antidote. In this study we show that host mutants with defects in either subunit of the ClpXP protease survive the loss of a plasmid that contains a P1 addiction module. The small antidote protein Phd is fully stable in these two mutant hosts, whereas it is labile in a wild-type host. We conclude that the role of ClpXP in the addiction mechanism of P1 is to degrade the Phd protein. This conclusion situates P1 among plasmids that elicit severe withdrawal symptoms and are able to do so because they encode both a cell toxin and an actively degraded macromolecule that blocks the synthesis or function of the toxin.**

Bacteriophage P1 lysogenizes *Escherichia coli* as a low copy number plasmid that is maintained with a loss frequency of about 10^{-5} per cell per generation (1). This remarkable stability is higher than can be accounted for by the mechanisms that ensure stringent control of plasmid replication and active partition of the replicas (2). The additional stabilization is provided by a "plasmid-addiction" module that selectively kills plasmid-free segregants or their progeny (3). The addiction module of P1 encodes two small proteins: Phd and Doc. Phd is responsible for *p*revention of *h*ost *d*eath in P1 lysogens; Doc causes *d*eath *o*n *c*uring. In cells that harbor a P1 prophage Phd must be maintained at a concentration sufficient to inhibit effectively the synthesis or the function of the toxin. We favor the view that Phd is an inhibitor of Doc function rather than an inhibitor of Doc synthesis. The latter hypothesis is difficult to reconcile with the apparent translational coupling of Doc synthesis to the synthesis of Phd (3) and with the delay of several generations after loss of the plasmid before withdrawal symptoms become manifest. On the other hand, if Phd is an antidote to Doc, then the death of cells that lose the plasmid could be most simply explained by the more rapid inactivation of the antidote compared to the toxin.

Lability of the macromolecule that prevents toxin synthesis or function accounts for the selective killing of cells cured of addicting plasmids such as R1 or F. The products of the *sok* gene of R1 and of its homologs in other plasmids are labile antisense RNAs subject to rapid degradation by nucleases (4), whereas the protein products of *ccdA* of F (5) and *pemI* of R100 (ref. 6; identical to *kis* of R1; see ref. 7) are actively degraded by the bacterial Lon protease. In the absence of *de novo* synthesis of these macromolecules, their degradation allows the toxins to be synthesized or released and to kill the cured cells.

Lon is one of two well-characterized ATP-dependent serine proteases of *E. coli*, the other being ClpAP (8). The Clp proteases form a family in which a proteolytic subunit, ClpP, is associated with one or another specificity subunit bearing the ATPase. Recently a specificity subunit was described that is the product of a gene in the *clpP* operon, *clpX* (9). ClpX was identified as the activator of ClpP for degradation of λO protein (9, 10).

We show here a requirement for the activity of the ClpXP bacterial protease in the expression of P1 plasmid addiction and provide evidence as to the identity of the relevant substrate of that protease.

MATERIALS AND METHODS

Bacterial Strains, Bacteriophages, and Plasmids. The bacterial strains and plasmids used in this work and their sources are listed in Table 1. Either tryptone broth (TB), Luria–Bertani medium (LB), or M63 minimal salts supplemented with glucose (14) was used to culture *E. coli*. The following antibiotics were added as appropriate: ampicillin, 100 μg/ml; spectinomycin, 25 μg/ml; kanamycin, 25 μg/ml; chloramphenicol, 25 μg/ml. P1Cm (15) and P1Km (16) lysogens were constructed by selection for their antibiotic-resistance markers, chloramphenicol and kanamycin, respectively.

Cloning Procedures and DNA Sequencing. Commercially prepared restriction endonucleases were used as specified by the suppliers. Plasmid DNA was prepared by a rapid alkaline lysis procedure and analyzed by gel electrophoresis using standard methods (17). Sequenase (United States Biochemical) and standard primers (Bethesda Research Laboratories) or appropriate primers synthesized on a MilliGen model 8750 oligonucleotide synthesizer were used for DNA sequencing on double-stranded template DNA (17).

Plasmid-Addiction Assay. Strains carrying either the pSC101-derived thermosensitive vector pGB2ts (13) or pGB2ts::*phd-doc* (Table 1) were used in this assay. Bacterial cultures were maintained in logarithmic growth phase at 30°C in TB supplemented with the appropriate antibiotics. The experiment was initiated by dilution of the cultures into antibiotic-free TB at 40°C (nonpermissive for vector replication). Subsequently, measurements of absorbance at 600 nm (A_{600}) and of colony-forming units (cfu) were made. The cultures sampled for absorbance readings were successively diluted so as to maintain $A_{600} < 0.25$. More dilute cultures, initially at $\approx 10^3$ cfu/ml, were sampled for plating on TB agar containing appropriate antibiotics and colonies were counted after incubation overnight at 30°C. Addiction was judged to have occurred when bacteria that lost pGB2ts::*phd-doc* failed to score as cfu.

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Abbreviation: cfu, colony-forming unit(s).
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Table 1. Bacterial strains and plasmids

Strain or plasmid*	Description/genotype	Source or ref.
E. coli strain		
TB1 (BR6424)	*ara*Δ(*lac proAB*) *rpsL* (φ80 *lacZ*ΔM15) *hsdR*	11
SG22025 (BR4726)	Δ*lac rcsA*166::mini-*kan* parent of the following two strains	S. Gottesman
SG22093 (BR4727)	Δ*lac rcsA*166::mini-*kan clpP*1::*cat*	S. Gottesman
SG22095 (BR4729)	Δ*lac rcsA*166::mini-*kan lon*-146::mini-Tn*10*	S. Gottesman
SG20250 (BR4749)	"Wild-type" relative of MC4100 and parent of the following four strains	9 and references therein
SG22098 (BR4750)	*clpP*1::*cat*	9
SG22099 (BR4751)	*clpA*319::mini-*kan*	9
SG22100 (BR4752)	*clpB*::mini-*kan*	12
SG22101 (BR4753)	*clpX*::*kan*	9
Plasmid		
pGB2ts (pG)	pSC101-based vector, thermosensitive for replication, Sp^r/Sm^r	13
PGB2ts::phd-doc (pG5)	Derivative of pGB2ts expressing *phd* and *doc*, Sp^r/Sm^r	3
pmalE::phd	Derivative of pMAL-c2 (New England Biolabs) expressing an in-frame MalE-Phd fusion protein, Ap^r	This work

*Names in parentheses are those assigned in this laboratory.

Purification of MalE-Phd. In the absence of an *in vitro* activity assay to monitor Phd, our purification was based on gene fusion technology (18). A DNA segment containing only the open reading frame of *phd* [positions 366–584 (3)] with a 14-bp 3′ extension (TAATGAGGATCCCC) was amplified by PCR. The 233-bp fragment thus generated was treated with polynucleotide kinase to phosphorylate the blunt ends and cloned into the *Xmn* I site of the expression vector pMAL-c2 (New England Biolabs). The *Xmn* I site had been introduced close to the 3′ terminus of the *E. coli malE* gene (New England Biolabs). The resulting construct, called p*malE*::*phd*, expressed an in-frame fusion of the *malE* and *phd* genes from the inducible *tac* promoter (19). The frame of the fusion and the intactness of the *phd* open reading frame were confirmed by nucleotide sequence analysis. Cells harboring this plasmid were grown in LB at 37°C to an A_{600} = 0.5. The expression of the fusion protein was then induced by the addition of isopropyl β-D-thiogalactoside to a final concentration of 0.3 mM. The cells were further incubated for 2 hr and harvested by low-speed centrifugation at 4°C. The pellet was resuspended in buffer A (10 mM Tris·HCl, pH 7.4/200 mM NaCl/1 mM EDTA/1 mM dithiothreitol) and the cells were broken by sonication [Braun (Melsungen, F.R.G.) Sonic U apparatus]. Soluble proteins were separated from the membrane fraction by centrifugation for 30 min at 10,000 × *g* at 4°C. The supernatant was loaded directly onto a column packed with an amylose resin (New England Biolabs) and equilibrated in buffer A. After washing the column with 4 column volumes of buffer A, proteins bound to the amylose resin were eluted by 10 mM maltose in buffer A. The eluted fusion protein was >95% pure as judged by inspection of Coomassie-stained SDS/12% (wt/vol) polyacrylamide gels (data not shown) and was used directly to raise polyclonal antibodies against MalE-Phd (Hazelton, Washington).

Turnover of Phd. Cultures of P1Cm lysogens of strains SG20250, SG22098, and SG22101 (Table 1) were grown in logarithmic phase in M63 glucose minimal medium at 37°C. Newly synthesized proteins were radiolabeled in a 2-min pulse by the addition of 10 μCi of [^{35}S]methionine (1000 Ci/mmol; 1 Ci = 37 GBq; Amersham) to the growth medium. The radiolabeled methionine was chased by dilution of the culture at 37°C with M63 glucose medium containing 0.5% unlabeled methionine. Aliquots removed at various times following the labeling were prepared for immunoprecipitation as described in Stout *et al.* (20). Phd was immunoprecipitated using polyclonal antibodies raised against MalE-Phd and analyzed on an SDS/20% (wt/vol) polyacrylamide gel. Gels were fixed in 10% acetic acid/5% glycerol for 2 hr, transferred to Whatman 3MM chromatography filter paper, dried at 80°C under vacuum, and exposed to a Kodak XAR-2 x-ray film for 72 hr. For each sample the measured amount of Phd was normalized with respect to the total amount of immunoprecipitated radiolabeled protein.

RESULTS

Involvement of ClpXP in P1 Plasmid Addiction. A wild-type *E. coli* strain carrying a plasmid that is thermosensitive for replication and contains the P1 *phd-doc* addiction module shows a striking addiction phenotype upon a shift to nonpermissive conditions (3). In the experiment of Fig. 1, the bacterial culture, at about 3 hr after a shift to 40°C, decelerated its increase in cell mass, measured by A_{600} (left upper panel), and ceased to show any further increase in cfu, scored by plating on nonselective agar at 30°C (left lower panel). Only cells that retained a copy of pGB2ts::*phd-doc* survived as colony formers, as shown by replica plating to selective agar (left lower panel). Fig. 1 also shows that an isogenic strain carrying a control plasmid that lacks a P1 addiction module loses the plasmid at the same rate but without an associated loss of bacterial viability.

With the aid of pGB2ts::*phd-doc*, we examined the potential role of host proteases in the expression of P1 plasmid addiction. We tested the effect of mutations in *lon* and *clpP* that eliminate the activity of ATP-dependent serine proteases of *E. coli* (8). The results of one of these experiments are shown in Fig. 1 (middle and right panels). The strain carrying a mutation

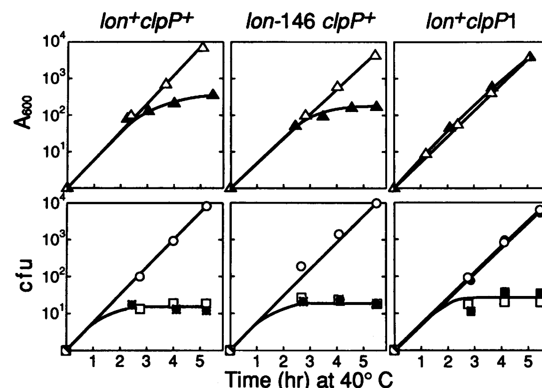

FIG. 1. Lethality upon loss of pGB2ts::*phd-doc* and its suppression in a *clpP* mutant strain. Experimental details of this typical experiment are given in the text. Bacterial strains used were SG22025, SG22093, and SG22095 (Table 1), initially carrying either pGB2ts (open symbols) or pGB2ts::*phd-doc* (closed symbols). △, Absorbance measurements; ○, cfu on nonselective medium; □, spectinomycin-resistant cfu which retained the parental plasmid.

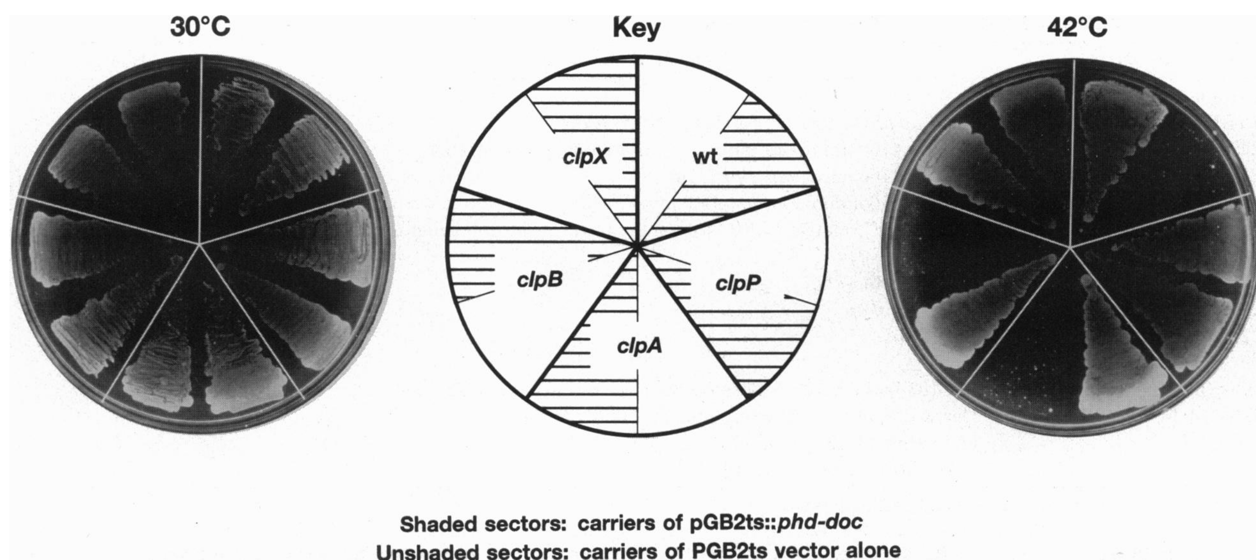

FIG. 2. Plasmid-addiction assay on agar. The bacterial strains SG20250, SG22098, SG22099, SG22100, and SG22101, carrying either pGB2ts or pGB2ts::*phd-doc* (Table 1), were grown in TB at 30°C. Aliquots of cultures in logarithmic growth phase were spread onto prewarmed plates of nonselective agar. Duplicate plates were incubated overnight at 30°C and 42°C.

in the *lon* gene was as addicted to pGB2ts::*phd-doc* as was the wild-type strain. In contrast, the strain carrying a mutation in *clpP* showed no growth arrest upon the loss of pGB2ts::*phd-doc*. The rate of loss of the normally addicting plasmid was not altered, indicating that the mutation in *clpP* does not interfere with the thermosensitivity of the plasmid. This result identifies *clpP* as a host function involved in the mechanism of P1 plasmid addiction.

The catalytic protease subunit ClpP can form functional proteases with different substrate specificities (8, 9), in association with the ATPase ClpA (21) or ClpX (9), and has been proposed (22) to do so with a homologous ATPase, ClpB (12). We tested mutants separately defective in each of the three ATPases for their viability following loss of pGB2ts::*phd-doc*. Fig. 2 shows that growth of the parent strain and of *clpA* and *clpB* derivatives was severely inhibited at 42°C. The *clpX* derivative survived the loss of pGB2ts::*phd-doc*, as did the *clpP* derivative. Essentially identical results were obtained when the above strains were analyzed in liquid culture (data not shown). It can be concluded that it is the ClpXP protease that plays a role in the addiction mechanism.

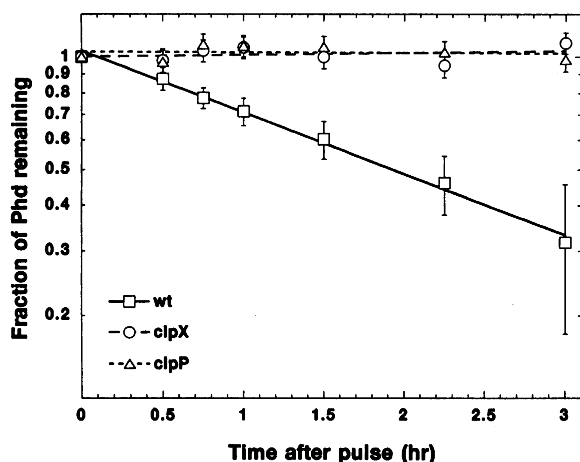

FIG. 3. *In vivo* degradation of Phd. Experimental details are given in the text. Sources of labeled Phd were P1 lysogens of parental strain SG20250, *clpP* mutant SG22098, and *clpX* mutant SG22101 (Table 1). Three independent sets of measurements were analyzed.

Evidence That Phd Is a Substrate for ClpXP *in Vivo*. The antidote protein, Phd, is the most likely product of the addiction module to be a substrate for a protease. To test the idea that the role of the ClpXP protease in P1 plasmid addiction is to assure the timely degradation of Phd, we examined the stability of Phd by pulse–chase experiments. The results of Fig. 3 show that Phd was not stable in the wild-type strain SG20250 but was degraded slowly with a half-life of 120 min. The generation time of SG20250 in minimal medium was 68 min and thus it took almost two generations for half of the labeled protein to be degraded. This observation suggests that the delay in Doc-induced loss of capacity for cell division until about four or five generations following plasmid loss (3) is primarily due to a delay before the toxin becomes active, rather than to a slow poisoning. Fig. 3 also shows that in either a *clpP* or *clpX* mutant background Phd was fully stable during the course of our experiments.

DISCUSSION

Plasmid addiction is a homeostatic mechanism that offsets disturbances to the genetic homogeneity of a bacterial population by programing the death of genetic variants. It is a response of last resort. The destructiveness involved makes it a particularly striking example of the paradoxical requirement for energy expenditure to maintain the status quo.[†] The mechanism can be extraordinarily simple. P1 plasmid provides only two gene products: toxin and antidote. The antidote must be actively destabilized to assure hereditary stability of the plasmid itself. The host provides the mechanism by which the requisite act of destabilization is accomplished, although the mechanism differs from case to case. In the example studied here, we have shown that proteolysis of the antidote, Phd, by ClpXP is likely to be essential for *E. coli* to become addicted to P1 plasmid. *In vitro* experiments with crude cell extracts or

†A requirement for energy expenditure to maintain the status quo is a paradox memorably rendered in ref. 23 by the Red Queen, who remarked to an exhausted Alice, with whom she had been running hand in hand: "Now, *here*, you see, it takes all the running *you* can do, to keep in the same place." The Red Queen, being a chess figure, does not have to exhaust herself in frantic homeostasis, which may, in some measure, account for her disdainful emphasis on "*you*."

purified proteins suggest that the action of ClpXP on Phd is direct and that Doc is not degraded by either ClpXP, ClpAP, or Lon, under assay conditions that allowed these three proteases to degrade λO (ref. 10; data not shown). The degradation of Phd frees the toxin or its target so that a lethal interaction between the latter two can proceed. Conceivably, ClpXP has an additional role in the addiction process—e.g., in a direct activation of the toxin or of the toxin's target—but there is no reason to believe this likely.

The turnover rate of Phd is slow relative to a number of rates of energy-dependent proteolyses reported in the literature (8, 24). This slow turnover (a half-life of almost two bacterial generations) minimizes the risk that variations in protease activity might lead to premature activation of Doc. A comparable rate of turnover was also found for the antidote protein, CcdA, of F (5). In contrast, the antisense RNAs of the *hok* killer gene family (25) are degraded rapidly (4). The risk that rapid degradation of the antisense RNAs entails is averted by a subsequent slow processing step, necessary to convert the translationally inactive full-length *hok* message into a 3′ truncated translationally active form (26–28).

Several homologs of plasmid-borne addiction genes have been found in the chromosome of *E. coli* (25, 29) and of distantly related bacterial species (30). Selective proteolysis such as reported here is a known component of numerous regulatory pathways, including those involved in development as well as homeostasis (8). In particular, members of the ClpP protease family are found in various animals, plants, and microorganisms (31). Taken together, these observations suggest that plasmid-addiction genes might have evolved from genes less destructive to the cell, progenitors of *doc*, *hok*, *ccdB*, and *pemK* that, in apparent contrast to the addiction genes, had biologically meaningful functions in their own presence.

The few substrates identified so far for the recently discovered ClpXP protease of *E. coli* (9) are all encoded by bacteriophages: the O replication protein of bacteriophage λ (298 amino acid residues) (10, 24), mutant forms of the bacteriophage Mu repressor protein (196 amino acid residues) (32), and the antidote protein Phd (73 amino acid residues) of bacteriophage P1. Phd, being the smallest of the three, would appear to be a promising substrate for studies of protease target specificity.

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1. Rosner, J. L. (1972) *Virology* **48,** 679–689.
2. Nordström, K. & Austin, S. J. (1989) *Annu. Rev. Genet.* **23,** 37–69.
3. Lehnherr, H., Maguin, E., Jafri, S. & Yarmolinsky, M. B. (1993) *J. Mol. Biol.* **233,** 414–428.
4. Gerdes, K., Nielsen, A., Thorsted, P. & Wagner, E. G. M. (1992) *J. Mol. Biol.* **226,** 637–649.
5. Van Melderen, L., Bernard, P. & Couturier, M. (1994) *Mol. Microbiol.* **11,** 1151–1157.
6. Tsuchimoto, S., Nishimura, Y. & Ohtsubo, E. (1992) *J. Bacteriol.* **174,** 4205–4211.
7. Bravo, A., de Torrontegui, G. & Diaz, R. (1987) *Mol. Gen. Genet.* **210,** 101–110.
8. Gottesman, S. & Maurizi, M. R. (1992) *Microbiol. Rev.* **56,** 592–621.
9. Gottesman, S., Clark, W. P., de Crecy-Lagard, V. & Maurizi, M. R. (1993) *J. Biol. Chem.* **268,** 22618–22626.
10. Wojtkowiak, S., Georgopoulos, C. & Zylicz, M. (1993) *J. Biol. Chem.* **268,** 22609–22617.
11. Johnston, T. C., Thompson, R. B. & Baldwin, T. O. (1986) *J. Biol. Chem.* **261,** 4805–4811.
12. Squires, C. & Squires, C. L. (1992) *J. Bacteriol.* **174,** 1081–1085.
13. Clerget, M. (1991) *New Biol.* **3,** 780–788.
14. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
15. Iida, S. & Arber, W. (1980) *Mol. Gen. Genet.* **177,** 261–270.
16. Fortson, M. R., Scott, J. R., Yun, R. & Vapnek, D. (1979) *Virology* **96,** 332–334.
17. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
18. Guan, C., Li, P., Riggs, P. D. & Inouye, H. (1987) *Gene* **67,** 21–30.
19. De Boer, H. A., Comstock, L. J. & Vasser, M. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 21–25.
20. Stout, V., Torres-Cabassa, A., Maurizi, M. R., Gutnick, D. & Gottesman, S. (1991) *J. Bacteriol.* **173,** 1738–1747.
21. Gottesman, S., Clark, W. P. & Maurizi, M. R. (1990) *J. Biol. Chem.* **265,** 7886–7893.
22. Kitagawa, M., Wada, C., Yoshioka, S. & Yura, T. (1991) *J. Bacteriol.* **173,** 4247–4253.
23. Carroll, L. (1865) *Alice in Wonderland*; reprinted (1992) by Norton, New York, 2nd Ed., p. 127.
24. Bejarano, I., Klemes, Y., Schoulaker-Schwarz, R. & Engelberg-Kulka, H. (1993) *J. Bacteriol.* **175,** 7720–7723.
25. Gerdes, K., Paulsen, L. K., Thisted, T., Nielsen, A. K., Martinussen, J. & Andreasen, P. H. (1990) *New Biol.* **2,** 946–956.
26. Thisted, T. & Gerdes, K. (1992) *J. Mol. Biol.* **223,** 41–54.
27. Thisted, T., Nielsen, A. K. & Gerdes, K. (1994) *EMBO J.* **13,** 1950–1959.
28. Thisted, T., Sørensen, N. S., Wagner, E. G. H. & Gerdes, K. (1994) *EMBO J.* **13,** 1960–1968.
29. Masuda, Y., Miyakawa, K., Nishimura, Y. & Ohtsubo, E. (1993) *J. Bacteriol.* **175,** 6850–6856.
30. Poulsen, L. K., Larsen, N. W., Molin, S. & Andersson, P. (1989) *Mol. Microbiol.* **3,** 1463–1472.
31. Gottesman, S., Squires, C., Pickersky, E., Carrington, M., Hobbs, M., Mattick, J. S., Dalrymple, B., Kuramitsu, H., Shiroza, T., Foster, T., Clark, W. P., Ross, B., Squires, C. L. & Maurizi, M. R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3513–3517.
32. Geuskens, V., Mhammedi-Alaoui, A., Desmet, L. & Toussaint, A. (1992) *EMBO J.* **11,** 5121–5127.