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Genes That Protect against the Host-Killing Activity of the E3 Protein of *Bacillus subtilis* Bacteriophage SPO1

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A cloned *rpoB* gene, specifying an apparently mutant RNA polymerase β subunit, protected *Escherichia coli* against the cytocidal effects of the E3 protein of bacteriophage SPO1, suggesting that RNA polymerase is the primary cellular target of the E3 protein. Two segments of the wild-type *E. coli* genome, one of which specifies a suppressor of *dnaK* mutations, and thus, possibly, a molecular chaperone, also provided protection when overexpressed, but wild-type *rpoB* did not.

Gene *e3* of *Bacillus subtilis* bacteriophage SPO1 specifies a cytotoxic protein that is believed to be involved in the shutoff of host biosyntheses. When the *e3* gene was expressed from an inducible promoter in uninfected cells of either *B. subtilis* or *Escherichia coli*, it caused the inhibition of DNA, RNA, and protein synthesis and, ultimately, cell death (30). RNA synthesis was shut off somewhat more rapidly than that of DNA or protein, suggesting that the effects on DNA and protein might be indirect results of the effect on RNA, but the primary target of E3's host shutoff activity has not previously been identified.

The cellular targets of several proteins that kill *E. coli* have been studied by selection of killing-resistant mutants. The most extensively studied is the CcdB (LetD) protein, which is specified by the F plasmid and which plays a role in plasmid maintenance by killing segregants that have lost the plasmid. Mutations conferring resistance to killing were found in genes specifying the DNA gyrase A subunit, the chaperonins GroES and GroEL, and two unknown proteins (2, 18, 19), and these results were interpreted as suggesting that the gyrase A protein is the primary target of CcdB and that the chaperonins are necessary either for the correct folding of CcdB or for its interaction with the gyrase. An *E. coli* mutation causing resistance to the *gefA* family of plasmid or chromosomal kill genes was located in a previously unknown *E. coli* gene (21), while mutants resistant to killing by the products of either the P gene or the *kil* gene of phage λ were isolated, but the genes affected were not identified (5, 16).

E3-resistant mutant DH5R. On the supposition that E3's cellular target was likely to be mutable to E3 resistance, we isolated E3-resistant mutants of *E. coli*. (*E. coli* was chosen rather than *B. subtilis* because of the more extensive information available about the *E. coli* genome, because of the greater stability of our E3 expression vector in *E. coli*, and because the effect of E3 on *E. coli* was at least as great as that on *B. subtilis* [30]). Cells of *E. coli* DH5, carrying plasmid pPW19-e3, which carries the *e3* gene under control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter (30), were plated on medium containing IPTG. Approximately 1 in 10^5 cells survived to form colonies, and the plasmids from 27 of these colonies were analyzed. Twenty-four plasmids differed from pPW19-e3, either in restriction digest pattern or in ability to cause killing when transformed into DH5 cells which were then plated on IPTG. These colonies presumably had survived because their plasmids had lost the ability to provide active E3. The other three colonies were cured of plasmids by growth in the absence of the selective antibiotic, and their E3 resistance was confirmed by showing that they survived when transformed by pPW19-e3 and plated on IPTG. One of these was chosen for analysis and was named DH5R.

All techniques not specified here were as described previously (30).

Identification of protective genes. To try to identify the gene(s) mutated in DH5R, we prepared two libraries of its genome in plasmid pACYC177 (3, 23), which is compatible with pPW19-e3. Chromosomal DNA was prepared by a modification of the procedure of Ausubel et al. (1). Aliquots were partially digested with 0.005 U of *Hpa*II per μl for 20 min (yielding DNA fragments whose sizes were mostly between 2 and 4 kb) or with 0.0025 U of *Hpa*II per μl for 30 min (yielding DNA fragments whose sizes were mostly between 4 and 10 kb). A 100-ng sample of the partially digested genomic DNA was ligated to 200 ng of *Cla*I-digested pACYC177. The ligation sample was precipitated with ethanol and redissolved in 4 μl of 5.0 mM Tris–0.5 mM EDTA, pH 7.4. One microliter was used to transform 20 μl of DH5(pPW19-e3) culture by electroporation (6), with selection for transformants on plates containing ampicillin (AMP), chloramphenicol (CM), and IPTG. (AMP and CM select for the presence of the pACYC177 derivative and pPW19-e3, respectively.) A similar library from DH5 was used in a parallel transformation. The *Cla*I cloning site in pACYC177 and all other cloning sites used in this work are within the Km^r gene, which is inactivated by the presence of an insert.

The frequency of IPTG-resistant transformants among all Amp^r Cm^r Km^s transformants ranged from 0.4×10^{-4} to 1.0×10^{-4}. Twenty-five of these IPTG-resistant colonies were tested to identify those whose E3 resistance was due to the pACYC177 derivative. The plasmids obtained from each clone were used to transform DH5, with separate selections for cells transformed with the Cm^r or Amp^r plasmid. Cm^r transformants were streaked onto plates with CM plus IPTG to identify the transformants whose pPW19-e3 had lost its lethality. Seven clones retained active Cm^r plasmid pPW19-e3, and four of these had Amp^r plasmids which conferred IPTG resistance upon DH5(pPW19-e3). These four represent clones of *E. coli* DNA fragments that protect against the killing effect of E3 and were named pPW40, pPW50, and pPW60 (from the DH5R libraries) and pPW70 (from the DH5 library). Thus, the fre-

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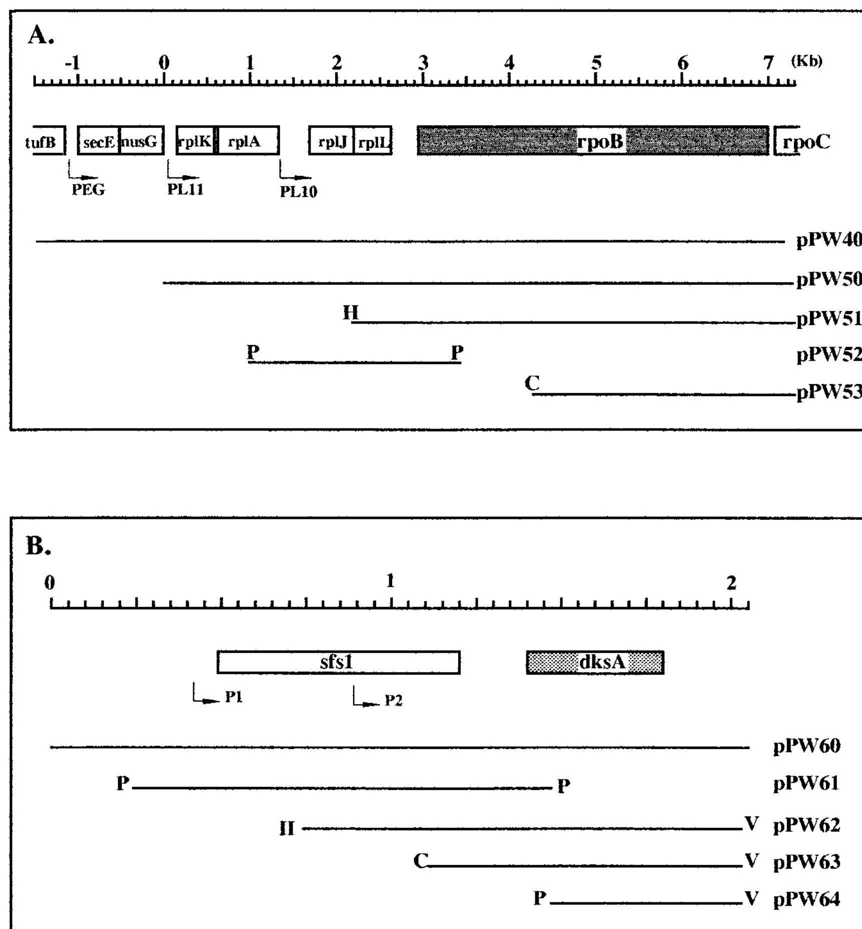

FIG. 1. Maps of the *rpoB* and *dksA* regions. In each panel, map distances in kilobases (top line), genes and promoters (boxes and arrows, respectively, below that line, with directions of transcription indicated by the arrows), the lengths of the cloned fragments relative to the map and the enzymes used in subcloning (lines with letters at the ends), and the name of each recombinant plasmid are indicated. P, *Pvu*II; C, *Cla*I; V, *Eco*RV; H, *Hin*dIII; II, *Hin*cII. All cloned fragments were inserted within the Km^r gene of pACYC177. (A) *rpoB* region (20). The 0-kb point represents the left end of the cloned fragment in pPW50 and corresponds to about 4,187.4 kb in EcoMap 7 (24, 25). Subfragments of pPW50 include the following: in pPW51, a 5.4-kb fragment extending from the *Hin*dIII site in *rplJ* to the *Hin*dIII site in the vector, inserted at the *Hin*dIII site of pACYC177; in pPW52, a 2.5-kb fragment, extending from a *Pvu*II site within *rplA* to a *Pvu*II site within *rpoB*, inserted at the *Sma*I site of pACYC177; and in pPW53, a 3.2-kb fragment, extending from the *Cla*I site within *rpoB* to the *Sma*I site in the vector, inserted between the *Cla*I and *Sma*I sites of pACYC177. (B) *dksA* region (9). The 0-kb point represents the left end of the cloned fragment in pPW60 and corresponds to about 162 kb in EcoMap 7 (24, 25). Subfragments of pPW60 include the following: in pPW61, a 1.2-kb *Pvu*II fragment, including all of gene *sfs1*, inserted at the *Sma*I site of pACYC177; in pPW62, a 1.3-kb *Hin*cII-*Eco*RV fragment, including all of gene *dksA*, inserted at the *Sma*I site of pACYC177; in pPW63, a 1.0-kb *Cla*I-*Eco*RV fragment, including all of gene *dksA*, inserted between the *Cla*I and *Sma*I sites of pACYC177; and in pPW64, a 0.5-kb *Pvu*II-*Eco*RV fragment, including most, but not the first 36 codons, of *dksA*, inserted at the *Sma*I site of pACYC177.

quency of cloned fragments which protect against E3, among all of the fragments in these genomic libraries, was approximately 10^{-5}.

The ends of the cloned fragments in plasmids pPW40, pPW50, pPW60, and pPW70 were sequenced, and the sequences were used to probe the GenEMBL database, in each case revealing virtual identity with a known *E. coli* sequence. pPW40 and pPW50 each include the *rpoB* gene (20) and several adjacent genes near 90 min on the *E. coli* chromosome (Fig. 1A). pPW60 includes the *sfs1* and *dksA* genes (9, 11) near 3.6 min (Fig. 1B), and pPW70 includes *smpB*, *ssrA*, and *slpA* (4, 12, 17, 22) at about 57.5 min. On EcoMap 7, the cloned fragments in pPW50, pPW60, and pPW70 extend approximately from 4187.4 to 4194.7, from 160.0 to 162.0, and from 2755.2 to 2759.2 kb, respectively (24, 25). The restriction map of each fragment was identical to that of the *E. coli* chromosome segment adjacent to the known sequence (13, 24, 25).

To identify specific genes responsible for the protective activities, the subclones indicated in Fig. 1 were tested for their capacity to protect against E3 activity. As shown in Fig. 2, the subclones which included the intact *rpoB* or *dksA* gene provided protection. All others did not. (pPW64 includes all but the first 36 codons of *dksA*, in frame with the Km^r gene, which may account for the slight protective activity that it appeared to provide.) The *rpoB* gene encodes the β subunit of RNA polymerase (20), while *dksA* specifies a dosage-dependent suppressor of *dnaK* mutations (9). Because most of the sequence of the fragment in pPW70 was not known until recently, we have not yet tried to identify the gene responsible there.

Since both *rpoB* and *dksA* were cloned from DH5R, the E3-resistant mutant, we supposed that one of the protective genes carried a mutation responsible for E3 resistance and that the other was a wild-type gene which was protective because of overexpression. The 4.0-kb wild-type *rpoB* gene, obtained from R. Landick (15), was cleaved from pRL385 on a 4.1-kb *Bam*HI-*Sac*I fragment, which was blunt ended by treatment

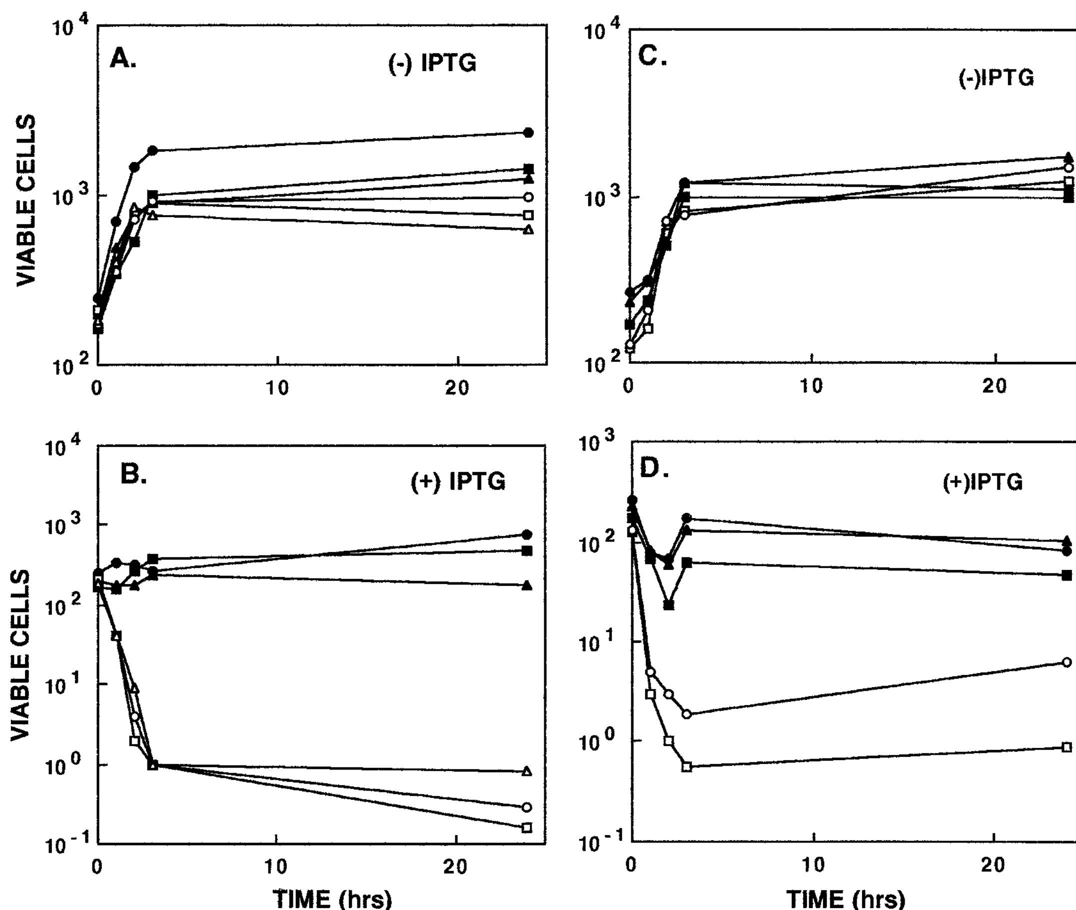

FIG. 2. Protection by the *rpoB* and *dksA* genes against E3-induced killing. Cultures of DH5(pPW19-e3), also containing pACYC177, pPW40, pPW50, or pPW60 or various subclones of pPW50 or pPW60, growing exponentially at 37°C in L broth plus CM and AMP, were each split into two cultures. At time zero, 0.2 mM IPTG was added to one culture of each pair, and at various times thereafter, aliquots were diluted and plated on Trypticase soy agar plates containing AMP and CM and lacking IPTG. (B and D) Cultures with IPTG; (A and C) cultures without IPTG. The graphs show the number of viable cells (10^6) per milliliter of undiluted culture as a function of time after IPTG addition. (A and B) ■, pPW40; ●, pPW50; ▲, pPW51; □, pPW52; ○, pPW53; △, pACYC177. (C and D) ■, pPW60; □, pPW61; ●, pPW62; ▲, pPW63; ○, pPW64.

with T4 DNA polymerase and inserted at the *Sma*I site of pACYC177. The 0.5-kb wild-type *dksA* gene, obtained from E. A. Craig (9), was cleaved from pJK538 on a 1.1-kb *Cla*I-*Bst*1107I fragment, which was inserted between the *Cla*I and *Sma*I sites of pACYC177. Each was inserted into the Km^r gene in the same orientation as the insert fragments in pPW50 and pPW60 and their subclones. The overexpressed wild-type *dksA* gene provided protection against killing by E3, but the wild-type *rpoB* gene did not. Thus, we believe that the selected *rpoB* gene has a mutation that makes it *e3* resistant, while the wild-type *dksA* gene depends on overexpression for its protective activity. The specific nature of the mutation in the cloned *rpoB* gene still needs to be determined, and its presence in DH5R has yet to be confirmed.

Interpretation. The most plausible interpretation of these data is that E3 acts upon the RNA polymerase, interfering with its ability to transcribe host DNA, and that the *rpoB* mutation alters the structure of the RNA polymerase so that it is resistant to the activity of E3.

Several additional arguments support this interpretation. (i) The sequence of a central region of the E3 protein shows remarkable similarity to the region of σ^{54} that is required for binding to *E. coli* RNA polymerase (29, 30). In each region, more than 50% of the amino acids are acidic; most of the rest are hydrophobic, with many of them spaced at 7-amino-acid intervals, and there are no basic amino acids. Mutation analysis showed that both the acidic and the hydrophobic amino acids were essential for binding of σ^{54} to RNA polymerase (29). Thus, the E3 amino acid sequence may be particularly suitable for binding RNA polymerase. (ii) RNA polymerase is a natural target for host shutoff mechanisms. Both T4 and T7 are known to have multiple mechanisms that target their host's RNA polymerase (7, 14). Host mutations causing resistance to the activity of the T4 *alc* gene product have in fact been located in the *E. coli rpoB* gene (26, 28). (iii) This interpretation explains the fact that net RNA synthesis is shut off somewhat more rapidly than DNA or protein synthesis when *e3* is expressed from an inducible promoter in uninfected cells (30).

The role of DksA is less obvious. The fact that overexpression of *dksA* suppressed a *dnaK* deletion mutant (9) suggests that DksA might have some of the activities of a molecular chaperone. If DksA does act as a chaperone, its role might be to prevent or reverse a change in RNA polymerase structure caused by E3. Support for that possibility comes from the fact that the DnaK protein is known to be capable of protecting *E. coli* RNA polymerase against heat denaturation and of reactivating the polymerase after it has been heat denatured (27). There is, however, no direct evidence that DksA acts as a

TABLE 1. Pulse-labeling of E3 protein[a]

Strain	IPTG concn (mM)	E3 band activity (normalized)[b]	*n*
DH5(pPW19-e3)	0.2	1.0	3
DH5R(pPW19-e3)	0.2	0.74 ± 0.35	3
DH5(pPW19-e3, pACYC177)	0.2	1.27 ± 0.34	4
DH5(pPW19-e3, pPW50)	0.2	0.80 ± 0.05	4
DH5(pPW19-e3, pPW60)	0.2	1.30 ± 0.23	2
DH5(pPW19-e3, pPW70)	0.2	1.19 ± 0.36	2
DH5(pPW19-e3)	0.02	0.61 ± 0.07	2
DH5(pPW19-e3, pACYC177)	0.02	0.72 ± 0.08	2
DH5(pPW19)	0	0.15 ± 0.01	2

[a] Cultures growing exponentially at 37°C in C6 medium plus CM (and AMP where appropriate) were induced with the indicated concentrations of IPTG. Ten minutes later, they were pulse-labeled for 2 min with 20 μCi of [^{35}S]methionine and [^{35}S]cysteine per ml. Protein extracts were subjected to electrophoresis, autoradiographs were prepared, and the total activity of each E3 band was measured by densitometry. The activity is defined as the intensity of the band, integrated over the entire area of the band, measured with a Molecular Dynamics computing densitometer.

[b] To correct for variations from experiment to experiment in time of exposure, the activity of each band was normalized to that for 0.2 mM IPTG-induced DH5(pPW19-e3) from the same experiment. In the rare cases for which that band was not appropriately available, the 0.2 mM IPTG-induced band from DH5(pPW19-e3, pACYC177) was used instead. Activities are given as means ± standard deviations for *n* independent determinations, each utilizing extracts prepared on different days from independently labeled cultures. The background at the E3 band position in the absence of E3 is shown by the DH5(pPW19) data.

chaperone, and there are other possible explanations for protection by DksA. For instance, it could act on E3, directly or indirectly, to prevent E3's activity, or it could protect the cells against the consequences of E3 action on RNA polymerase.

We do not know which gene is responsible for pPW70's protective activity or whether it is sense or antisense expression that is important (unlike all of the other genes cloned in pACYC177, the genes in pPW70 are oriented in the opposite direction from the Km^r gene) and thus are not in a position to offer specific interpretations.

The simplest alternative to any of the above interpretations is that one or another of the protective genes might protect by interfering with the synthesis of E3. To rule out that interpretation, we measured the rate of E3 synthesis in each strain by pulse-labeling followed by gel electrophoresis, autoradiography, and densitometry. Table 1 shows that neither pPW60 nor pPW70 caused any decrease in E3 synthesis. In each strain in which the mutant *rpoB* gene was present [in DH5R(pPW19-e3) and in DH5(pPW19-e3; pPW50)], there was a small decrease which was observed repeatedly, although it was not statistically significant. However, that decrease was not great enough to account for the absence of killing. A similar decrease could be caused in the wild-type strain [DH5(pPW19-e3)] by induction with a lower concentration of IPTG (Table 1), and that concentration was still effective at killing (Fig. 3).

The E3 protein apparently decreased the stability, although not the synthesis, of *e3* mRNA (30). As a hypothesis by which to explain all of the effects of E3 by a single mechanism, we suggest that the effect of E3 on RNA polymerase may be to cause it to terminate transcription prematurely, so that the stem-loop structure which is part of the normal termination signal is not completely transcribed and therefore is not available to serve its usual function of protecting the mRNA against excessively rapid degradation (8). Such a function for E3 would be superficially similar to that of the T4 Alc protein (10), although the amino acid sequence of E3 shows no significant similarity to that of Alc or of any prokaryotic protein, except

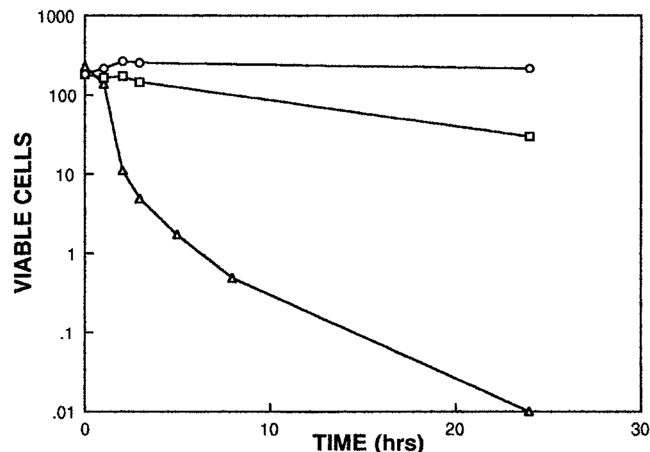

FIG. 3. Killing by lesser quantities of E3. Cultures of DH5(pPW19-e3) or DH5R(pPW19-e3) growing exponentially at 37°C in C6 plus CM (the same medium used for the pulse-labeling in the experiment whose results are shown in Table 1) were induced with various concentrations of IPTG. At intervals thereafter, aliquots were plated on CM plates to determine the number of surviving cells. The curves show the numbers of viable cells (10^6) per milliliter of undiluted culture. △, DH5(pPW19-e3) with 0.02 mM IPTG; ○, DH5R(pPW19-e3) with 0.02 mM IPTG; □, DH5R(pPW19-e3) with 0.2 mM IPTG.

σ^{54}, as discussed above. Significant similarities to several eukaryotic proteins have been mechanistically uninformative (30).

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