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Characterization of *degP*, a Gene Required for Proteolysis in the Cell Envelope and Essential for Growth of *Escherichia coli* at High Temperature

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The *degP* gene, required for proteolysis in the cell envelope of *Escherichia coli*, maps at approximately 3.5 min on the chromosome. Null mutations in *degP* result in temperature-sensitive growth. In certain genetic backgrounds, expression of abnormal periplasmic or inner membrane proteins (protein fusions or proteins with internal deletions) enhances the temperature-sensitive phenotype. Such growth defects were used as a selection for cloning the *degP* gene into Mud4042 and pACYC184 plasmid vectors, and a restriction map was determined. Analysis of deletion and insertion mutations on one of these plasmids showed that the *degP* gene is approximately 1.5 kilobases in size. The plasmid-encoded DegP protein had an apparent molecular weight of 50,000, as determined by maxicell analysis. Protein fusions between DegP and alkaline phosphatase had high alkaline phosphatase enzymatic activity, indicating that DegP is a periplasmic or membrane protein.

In *Escherichia coli* as well as other organisms, proteolytic enzymes function in at least three processes: (i) proteolytic modifications to produce mature proteins or to alter protein activity, (ii) turnover of "abnormal" proteins (e.g., protein fragments, missense proteins, and unfolded proteins), and (iii) turnover of "normal" (i.e., native or functional) proteins (16). Interference with these processes may cause growth defects due to failure to produce the mature, modified forms of specific proteins; failure to destroy potentially toxic abnormal polypeptides; or failure to control the levels of normally labile proteins.

We recently initiated genetic studies on the role of proteolysis in the cell envelope of *E. coli*. Proteolytic events in this compartment of the cell include processing of signal sequences for exported proteins and turnover of abnormal proteins (4, 11, 14). Much less evidence is available for turnover of native functional proteins. To examine the consequences of failure to carry out proteolytic events, we have devised a strategy for isolating mutants lacking proteolytic enzymes and have examined their phenotypes. Previously we described a screen for mutants defective in the degradation of a fusion protein located in the inner membrane of *E. coli*. One mutant allowed us to define a gene, *degP*, which appears to control synthesis of a cell envelope protease. Mutations in *degP* prevent the degradation of certain periplasmic fusion proteins and mutant forms of maltose-binding protein (21). Since that time, a number of other ordinarily unstable cell envelope proteins have been shown to be stabilized by the *degP* mutation. These include fusions between protein A and murine interleukin 2, human interleukin 2, and an 8-kilodalton (kDa) peptide secreted by T cells (G. Zurawski, personal communication). Thus, it appears that the protease controlled by *degP* plays a major role in degradation of proteins exported beyond the cytoplasm.

Indications of a role for the *degP* product in normal cell physiology came from preliminary growth studies of *degP* strains carrying genes for unstable cell envelope proteins. These strains were inviable under certain conditions (21). In the study reported here we studied this phenomenon in more detail. We found that *degP* strains grow poorly or are inviable at high growth temperatures. This growth phenotype suggests that *degP* is required for processing or degradation of endogenous *E. coli* proteins under these growth conditions.

The growth phenotype exhibited by *degP* strains provided a convenient test for *degP* function in cells, which we have exploited for genetic characterization of the locus. We have identified the *degP* gene and its gene product. Studies with alkaline phosphatase (AP) fusions to the *degP* gene product suggest that it is a cell envelope protein, possibly localized to the periplasmic space.

MATERIALS AND METHODS

Media and chemicals. Standard growth media have been described elsewhere (17). TYE agar is the same as LB agar except that it contains 8 g of NaCl per liter. Ampicillin (Amp; 200 μg/ml), kanamycin (Kan; 40 μg/ml, or 200 μg/ml in some experiments to select Tn*phoA* insertions onto pACYC184-derived plasmids), tetracycline (Tet; 20 μg/ml), and chloramphenicol (Cam; 25 μg/ml) were used as needed. XP (5-bromo-4-chloro-3-indolyl phosphate) was obtained from Bachem Fine Chemicals (Torrance, Calif.). Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs, Inc. (Beverly, Mass.).

Bacteria, bacteriophages, and plasmids. Bacterial strains and plasmids are listed in Table 1. Bacteriophages Mu *cts*62, Mu$^+$, and P1 (17, 20) were used in this study. Standard techniques for bacterial strain constructions were used (6, 17, 20). The *malT1*(Con) allele was introduced by cotransduction with *zhf*::Tn*10* or by first selecting a lambda *vir*-resistant, Mal$^-$ strain and subsequently transducing that strain to Mal$^+$ by using a P1 lysate grown on a *malT1*(Con) strain. The *recA1* allele was introduced by cotransduction

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype	Reference or source
Strain		
KS272	F⁻ Δ*lacX74 galE galK thi rpsL* (*strA*) Δ*phoA* (*Pvu*II)	21
KS303	KS272 *lpp-5508*	21
KS300	KS272 *recA1*	21
KS306	KS272 *lpp-5508 recA*	This study
KS330	KS272 *lpp-5508 degP4*::Tn*5*	21
KS340	KS272 *degP4*::Tn*5 zad-340*::Tn*10*	This study
KS373	KS272 *lpp-5508 gal*⁺ (Mu⁺)	This study
KS378	KS272 *lpp-5508 gal*⁺ *degP4*::Tn*5* (Mu⁺)	This study
KS419	KS272 *lpp-5508 gal*⁺ *malT*(Con) *zad-339*::Tn*10 degP4*::Tn*5* (Mu⁺)	This study
KS434	KS272 *lpp-5508 degP4*::Tn*5 recA1*	This study
KS464	KS272 *degP4*::Tn*5*	This study
KS470	KS272 *lpp-5508 gal*⁺ *malT*(Con) *degP4*::Tn*5 recA1*	This study
KS474	KS272 *degP41* (Δ*Pst*I-Kan^r)	This study
KS478	KS272 *zad-339*::Tn*10*	This study
AB354	*panD2 thr-1 leuB6 thi-1 lacZ4 rpsL8 supE44*	12
CG86	F⁻ Δ(*ara-leu*)*7697 lacX74 galE galK thi rpsL* (*strA*) *zaj*::Tn*10* (Mu⁺) pEG109	C. Gardel
CP78	*thr-1 leuB6 his-65 argH46 thi-1 ara-13 gal-3 malA1 xyl-7 tonA2 supE44*	B. Bachmann
M8820	F⁻ *araD139* Δ(*araCOIBA-leu*) *7697* Δ(*proAB argF lacIPOZYA*)*XII rpsL* (*strA*)	J. Beckwith
CA7027	HfrHayes Δ*lacU169*	J. Beckwith
XA7028	*ara-714 thr* Δ(*lac-pro*)*XIII rpsL* (*strA*)	J. Beckwith
Plasmid		
pACYC184	Cam Tet	5
pUC-4K	Kan with flanking multilinkers	23
pEG109	Mud4042(Amp)::*phoA proC*	9
pKS1	Amp Tsr-AP 1	21
pKS3	Amp Tsr-AP 2	21
pKS12	Cam *degP*	This study
pKS13	Cam *degP*	This study
pKS14	pKS12 Δ*Eco*RV-*Eco*RV	This study
pKS15	pKS12 Δ*Eco*RV-*Stu*I	This study
pKS16	pKS12 Δ*Pst*I-*Pst*I	This study
pKS17	pKS12 Δ*Sal*I-*Stu*I	This study
pKS19	pKS12 (*degP41* Δ*Pst*I-Kan^r)	This study

with *srl*::Tn*10*. The genetic nomenclature has been described elsewhere (1).

Monitoring protein degradation in bacterial strains. The protein degradation phenotype of *degP* strains can be determined by two methods. The first method (the AP halo assay) qualitatively monitors degradation of the labile Tsr-AP 2 protein in bacterial colonies growing on agar plates (21). The second method (osmotic shock and AP assay of strains expressing Tsr-AP 2) is a quantitative measure of the breakdown of this protein (21).

Cloning of *degP*. Standard DNA preparations and manipulations were used (6, 13). Plasmid libraries made with MudII4042 (9) were isolated as follows. pEG109 (MudII 4042::*phoA proC*) was not desirable as the MudII4042 parent plasmid because it carries the *phoA* gene and could interfere with testing of phenotypes associated with degradation of Tsr-AP 2. Therefore we first isolated MudII4042::*proAB* plasmids (pKS5, pKS6, pKS7, and pKS8) by inducing CG86 (a Mu *c*ts62-pEG109 lysogen) and infecting M8820(Mu *c*ts62) with selection for Pro⁺. Plasmid libraries were prepared by inducing M8820(Mu *c*ts62)-pMudII4042::*proAB* strains and were used to infect KS419 [*degP lpp malT*(Con) (Mu⁺)]. Strains able to grow at 42°C were selected on TYE-Cam plates. To test degradation of Tsr-AP 2, KS378 [*degP* (Mu⁺)] was transformed first with the Mud4042 plasmids and subsequently with pKS3 (encoding Tsr-AP 2). We found it difficult to transform MudII4042 plasmids into strains already containing pKS3.

Transposon mutagenesis of pKS17. Pho⁻ transpositions of Tn*phoA* into pKS17 were selected by infecting lambda::Tn*phoA* (10) into KS306(pKS17) and plating on TYE-Cam-Kan. Kan^r colonies were pooled, and plasmid DNA was used to transform KS306 to Kan^r. Individual clones were purified, and plasmid DNA was tested for complementation of the *degP* growth defect in KS470. Analysis of *Eco*RV digests differentiated between Tn*phoA* insertions into pACYC184 sequences (the 3.5-kilobase [kb] fragment) and chromosomal insert sequences (the 0.48-, 0.13-, 1.15-, 1.8-, and 0.72-kb fragments). All five Tn*phoA* insertions that inactivate *degP* lay in the 1.15- or 1.8-kb *Eco*RV-*Eco*RV fragments. All Tn*phoA* insertions into these fragments were mapped more precisely by *Eco*RV and *Dra*I single and double digests.

Pho⁺ Tn*phoA* insertions into pKS17 were selected in the same manner, except that TYE-Cam-Kan-XP was used for identifying Pho⁺ colonies. Analysis of *Eco*RV digests differentiated between Tn*phoA* insertions into pACYC184 sequences (the 3.5-kb fragment) and chromosomal insert sequences. Pho⁺ insertions were found in three small *Eco*RV fragments (0.48, 1.15, and 1.8 kb) and were mapped by *Eco*RV and *Dra*I single and double digests. Some insertions were also mapped by *Bam*HI and *Dra*I single and double digests. A single Pho⁺ insertion into the 0.48-kb fragment mapped in the *tet* gene of pACYC184.

Transfer of *degP* mutations from plasmids to the chromosome. The *degP41* mutation (Δ*Pst*I-Kan) was constructed and transferred to the chromosome as follows. pKS12 and pUC-4K were digested with *Pst*I, mixed, ligated, and transformed into KS306 with selection for Cam^r and screening for Cam^r Kan^r. Plasmids were isolated and analyzed from Kan^r colonies. pKS18 (*degP44*) and pKS19 (*degP41*) contain the 1.5-kb fragment of pUCK-4 and the 10-kb fragment from pKS12. These two fragments are ligated in opposite orientations in the two plasmids.

To recombine *degP41* and *degP44* onto the chromosome, pKS18 and pKS19 were transformed into CA7027 (HfrH). Resulting strains were mated to XA7028 [*ara thr* Δ(*lac-pro*) *str*] with selection for Thr⁺ Pro⁺ Kan^r Str^r. At least 50 exconjugants were pooled and used to prepare a P1 lysate. This P1 lysate was used to transduce KS272 (*degP*⁺) to Kan^r. Kan^r transductants were screened for Cam^r and growth at 42°C on TYE agar.

Pho⁺ *degP*::Tn*phoA* insertions were recombined onto the chromosome from pKS17 (*degP*::Tn*phoA*) plasmids in the same manner as *degP41*, except that the final P1 transduction was performed on TYE-XP-Kan agar. Blue (Pho⁺) colonies were purified and screened for Cam^r and growth at 42°C.

Pho⁻ *degP*::Tn*phoA* insertions were recombined onto the chromosome from pKS17 (*degP*::Tn*phoA*) plasmids in the same manner as *degP41*. However, because Tn*phoA* can

TABLE 2. Growth of *degP lpp* strains expressing abnormal proteins

Protein (half-life in *degP*$^+$)[a]	Growth[b] of *degP*$^+$ at:		Growth[b] of *degP4*::Tn*5* at:	
	30°C	42°C	30°C	42°C
None	+++	+++	+++	++
Tsr-AP 1 (stable)	+++	++	+++	+/−
Tsr-AP 2 (15 min)	+++	+++	+++	+/−
MBP-AP 15-1 (15 min)	+++	+++	+++	+/−
MBP-AP 18-1 (15 min)	+++	+++	+++	+/−
Bla-AP (30 min)	+++	+++	+++	++
MBP 57-142 (7 min)	+++	+++	+++	+/−
MBP 116 (20 min)	+++	+++	+++	++
MBP 57-142,SS18-1[c]	+++	+++	+++	+++
MBP 116,SS19-1[c]	+++	+++	+++	+++
MalF-AP (stable)	+++	+++	+++	−

[a] All strains are KS303 or KS330 containing plasmids encoding the proteins listed. MalF-AP is fusion J described in reference 4 encoded on a derivative of pDHB332. Other proteins and plasmids have been described elsewhere (8, 21).

[b] Growth was tested by streaking strains on TYE agar for single colonies. +++, Wild-type growth; uniformly sized, healthy colonies indistinguishable from an isogenic *degP*$^+$ strain. ++, Uniformly sized colonies, healthy in appearance but smaller than an isogenic *degP*$^+$ strain. +/−, Nonuniformly sized colonies; generally thin growth with faster-growing papillae appearing in confluent regions of a streak. −, No growth.

[c] SS18-1 and SS19-1 are signal sequence mutations that prevent export of MBP from the cytoplasm (3).

transpose during transduction, some Kan^r transductants are expected to be new Tn*phoA* transposition events rather than *degP*::Tn*phoA* recombinants. In the case of Pho$^-$ insertions, the new transpositions cannot be easily distinguished from *degP*::Tn*phoA* recombinants by AP production, but the two types of transductants can be distinguished by testing for linkage of Kan^r to the *degP* region or by testing for failure to grow on TYE agar at 42°C (a *degP* phenotype). In the final P1 transduction we observed far more transpositions of Tn*phoA* than recombination of *degP*::Tn*phoA*. Consequently it was not possible to identify *degP*::Tn*phoA* recombinants nonselectively at 30°C with screening by P1 transduction for linkage of Kan^r to *degP*. We therefore identified *degP*::Tn*phoA* recombinants by replica plating and screening for failure to grow at 42°C on TYE agar. Among Kan^r transductants, temperature-sensitive strains were identified at frequencies of 0/150 for *degP9-1*::Tn*phoA*, 2/150 for *degP10-2*::Tn*phoA*, 9/150 for *degP13-1*::Tn*phoA*, 6/150 for *degP16-1*::Tn*phoA*, and 8/150 for *degP18-1*::Tn*phoA*. P1 transductions confirmed that these strains contained Tn*phoA* insertions linked to *zad-339*::Tn*10* (80% linked to *degP*).

AP assay and osmotic shock. Procedures for AP enzyme assays and preparation of periplasmic proteins by osmotic shock have been described elsewhere (14, 21).

Labeling, immunoprecipitation, and polyacrylamide gel electrophoresis. Procedures for labeling, immunoprecipitating, and visualizing proteins have been described previously (21).

RESULTS

Growth defects of *degP* strains. As reported previously (21), in the *lpp-5508* strain background in which it was isolated, the *degP* mutation is not lethal to cells. *lpp-5508 degP4*::Tn*5* strains were viable at all temperatures tested, although *degP* colonies were somewhat smaller than wild-type colonies at 42°C (Table 2). In contrast, an isogenic *lpp*$^+$ *degP* strain did not form single colonies on TYE agar at 42°C, although a significant amount of cell material formed in the heavy region of a streak where one would expect confluent growth if the strain were viable. Growth on TYE plates was normal at 30 or 37°C. In LB liquid medium a *degP lpp*$^+$ culture that was shifted from 30 to 42°C contained normally shaped motile cells until 4 or 5 h after the shift, at which time misshapen nonmotile cells began to appear. Cessation of growth at 42°C thus appeared to occur slowly.

Although *degP lpp* strains are viable at 42°C, the expression at high levels of some abnormal periplasmic or membrane proteins made such strains temperature sensitive (Table 2). The ability of abnormal proteins to inhibit growth of *degP lpp* strains did not show a strong correlation with the degree to which these proteins are unstable in *degP*$^+$ strains. Two stable proteins, Tsr-AP 1 and MalF-AP, were toxic only to *degP*, not *degP*$^+$, strains. In addition, a *malT*(Con) mutation, which causes constitutive expression of the maltose regulon genes (7), including genes for several membrane and periplasmic proteins involved in maltose transport, caused *degP lpp* strains to be inviable at high temperature. We exploited this growth defect to facilitate characterization of the *degP* locus. Further experimental results presented below strengthen the correlation between the protein degradation and growth phenotypes associated with *degP* mutations.

Map position of *degP*. To facilitate genetic mapping of *degP*, Tn*10* insertions cotransducible with *degP* were isolated. A mixed culture of random Tn*10* insertion mutants was used as the donor in a P1 transduction with a *degP4*::Tn*5 lpp-5508*(pKS3) (encoding Tsr-AP 2) strain as the recipient. Tet^r colonies were selected at 37°C; most transductants grew poorly (the growth defect of *degP* strains). Six faster-growing transductants were isolated. These strains were able to degrade the Tsr-AP 2 fusion protein (as determined by the halo plate assay) and contained Tn*10* insertions linked by P1 transduction to the Tn*5* insertion in the *degP* gene (data not shown).

A Tn*10* insertion 50% linked to *degP* (*zad-340*::Tn*10*) was introduced into Hfr strains with origins of transfer in various regions of the *E. coli* chromosome. Matings were conducted selecting for Tet^r. HfrH (clockwise transfer from 97 min) and HfrP4 (counterclockwise transfer from 7 min) were found to transfer Tet^r at high frequency, indicating that the Tn*10* insertion is transferred as an early marker by these Hfr strains. P1 transductions with markers in this region showed that *degP4*::Tn*5* is 75% (132/175) linked to *pan* (3.4 map units) and 17% (35/197) linked to *fhuA* (3.7 map units).

Cloning of the *degP* gene. To isolate a recombinant plasmid carrying the *degP* gene, a plasmid library generated in vivo with the Mud4042 vector (9) was screened for plasmids which allow a *degP lpp malT*(Con) strain to grow at 42°C (see Materials and Methods). Plasmids that complemented the growth defect were isolated and tested to determine if they also allow Tsr-AP 2 to be degraded. Of 13 plasmids that allowed growth of the *degP* strain at 42°C, 3 (pKS9, pKS10, and pKS11) also allowed the fusion protein to be degraded (Table 3 and data not shown). All three plasmids contained common restriction fragments not present in the Mud4042 vector (0.95- and 1.35-kb *Pst*I-*Pst*I fragments), which indicated that all three plasmid inserts were derived from the same region of the chromosome. The other 10 plasmids which complement (or suppress) the growth defect but not the protein degradation defect may be acting by affecting the *malT*(Con) character necessary to observe the growth defect

TABLE 3. Degradation of Tsr-AP 2 in plasmid-bearing and mutant strains[a]

Strain	AP (%)	
	Periplasm	Membrane + cytoplasm
KS373[pKS3 (Tsr-AP 2)]	54	46
KS378(pKS3)	4	96
KS378[pKS3, pKS5 (MudII4042::*proAB*)]	6	94
KS378[pKS3, pKS10 (MudII4042::*degP*)]	65	35
KS306(pKS3, pACYC184)	66	34
KS434(pKS3, pACYC184)	2	98
KS434(pKS3, pKS12)	62	38
KS434[pKS3, pKS16 (Δ*Pst*I-*Pst*I)]	12	88
KS434[pKS3 pKS17 (Δ*Sal*I-*Stu*I)]	63	37
KS272 (*degP*$^+$)	59	41
KS464 (*degP4*::Tn*5*)	5	95
KS474 [*degP41* (Δ*Pst*I-Kan)]	7	93
KS516 (*degP10-2*::Tn*phoA*)	3	97
KS517 (*degP13-1*::Tn*phoA*)	4	96
KS518 (*degP16-1*::Tn*phoA*)	8	92
KS519 (*degP18-1*::Tn*phoA*)	6	94

[a] All strains were transformed with pKS3 (Tsr-AP 2), grown overnight at 30°C in LB-Amp medium (plus Cam as needed), diluted 1/50 into fresh LB-Amp (plus Cam), and grown to mid-log phase in LB-Amp at 37°C. Osmotic shock and AP assay procedures have been described elsewhere (14, 21). KS306 and KS373 are *degP*$^+$. KS378 and KS434 are *degP4*::Tn*5*. See Table 1 for full genotype.

in the host strain or by overexpressing some other protease capable of reversing the growth defect but not able to degrade Tsr-AP 2. These possibilities have not been pursued.

Because the Mud4042 vector in these plasmids is large (16.7 kb), we subcloned the *degP* insert into pACYC184 to facilitate restriction mapping. An 8-kb *Bam*HI-*Bam*HI fragment from pKS10 cloned into the *Bam*HI site of pACYC184 retained *degP* activity. In plasmids pKS12 and pKS13 this fragment was present in opposite orientations. pKS12 did not deleteriously affect the growth of strains in which it was present; in contrast, pKS13 caused both *degP*$^+$ and *degP* strains to grow slowly. Both pKS12 and pKS13 complemented the growth defect of *degP* strains. pKS12 was shown to complement the protein degradation defect of *degP* (Table 3). A restriction map of pKS12 is shown in Fig. 1. Several deletions were constructed on this plasmid by using convenient restriction sites (Fig. 1). Only one of the resulting plasmids, pKS17, was able to complement *degP* for both growth at high temperature and degradation of Tsr-AP 2 (Table 3). The other three plasmids (pKS14, pKS15, and pKS16) complemented neither the growth defect nor the protein degradation defect (Table 3 and data not shown).

Construction of a *degP* deletion and transfer to the chromosome. To confirm that the DNA insert in pKS12 does correspond to the chromosomal *degP* locus, a mutation constructed on this plasmid was recombined onto the chromosome and its map position was determined. The *Pst*I-*Pst*I deletion (Fig. 1) is internal to the DNA insert of pKS12 and therefore should retain homology to allow recombination with the chromosome. To provide a selectable marker, a DNA cassette containing a gene for kanamycin resistance with flanking *Pst*I sites (from pUCK-4 [18, 23]) was substituted for the two small *Pst*I-*Pst*I fragments of pKS12. The resulting plasmid contained a new *degP* allele, *degP41* (Δ*Pst*I-Kan).

The *degP41* allele was recombined from the plasmid onto the chromosome as described in Materials and Methods. In the final step of this procedure, Kan^r transductants were selected and scored for inheritance of the plasmid and for *degP* phenotypes. Among 12 Kan^r transductants, we detected 2 Cam^r strains that retained the plasmid and 10 Cam^s strains from which the plasmid had been lost. The 2 Kan^r Cam^r transductants grew normally at 42°C (a *degP*$^+$ phenotype), while all 10 Kan^r Cam^s transductants were unable to form single colonies at 42°C (a *degP* phenotype). In both Kan^r Cam^s transductants tested, Kan^r was linked by P1 transduction to a Tn*10* insertion 80% linked to *degP*. In both of these strains Tsr-AP 2 was not degraded (Table 4 and data not shown). Thus the *degP41* mutation, originally constructed on pKS12, when present on the *E. coli* chromosome, mapped in the same position as the original *degP4*::Tn*5* mutation and conferred the same growth phenotype and protein degradation phenotype as *degP4*::Tn*5*. By all criteria tested, pKS12 does contain the *degP* gene.

Transposon mutagenesis of *degP*. To determine more precisely the location of *degP* on pKS12 and pKS17, the positions of insertion mutations which inactivate the *degP* gene were determined. Many Tn*phoA* insertions were obtained (Fig. 2A). Only five of these insertions inactivated the *degP* gene on pKS17. All five DegP$^-$ insertions mapped to the left end of the chromosomal insert shown in Fig. 2.

DegP-AP protein fusions. If the *degP* gene product is the protease which is missing in *degP* strains, then the DegP

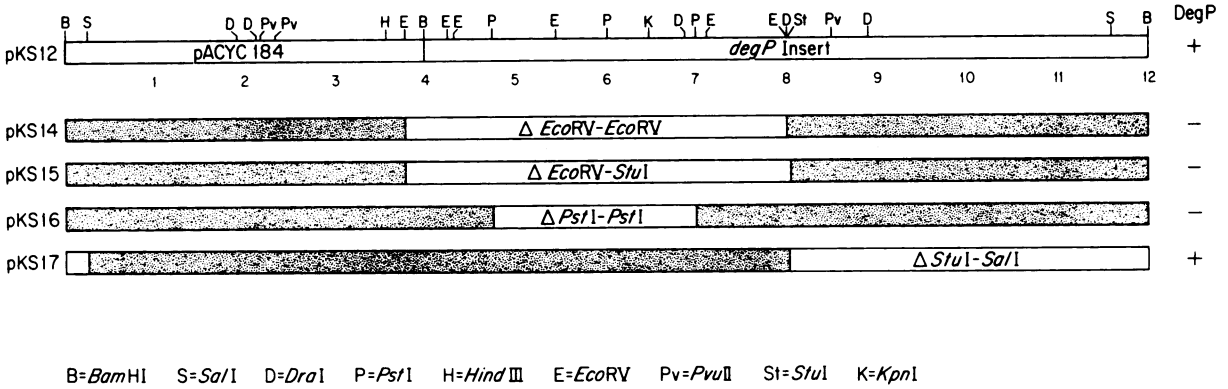

FIG. 1. Restriction map of plasmids carrying the *degP* gene. In pKS14, pKS15, pKS16, and pKS17 the stippled bars represent DNA present and the open bars represent DNA absent in each plasmid. The column headed DegP indicates the complementation behavior of each plasmid.

TABLE 4. DegP-AP protein fusions

degP::Tn*phoA* allele	Insertion site (kb)[a]	DegP-AP size (kDa)[b]	AP (U)[c]	
			Plasmid	Chromosome
35-1	5.89	49	1,121	121
39-1	5.89	49	1,140	134
37-2	5.84	51	934	134
36-1	5.82	51	1,069	129
43-1	5.74	53.5	1,269	117
44-2	5.33	66	354	67
42-1	4.83	96	488	57
38-2	4.73	97.5	493	74

[a] Insertion site refers to the position in kilobases on the restriction map of pKS12 shown in Fig. 1.
[b] DegP-AP protein sizes taken from Fig. 4.
[c] Plasmid-encoded AP activity was measured in strains KS300[pKS17 (*degP*::Tn*phoA*)]. Chromosome-encoded AP activity was measured in strains KS272 *srl*::Tn*10 recA1 degP*::Tn*phoA*(pKS17). AP activity was measured in mid-log-phase growing in LB-Cam medium at 37°C.

protein should be present in the periplasm or membrane (the compartment in which *degP* mutations stabilize abnormal proteins). To determine whether the *degP* gene codes for a periplasmic or membrane protein, we used the transposon Tn*phoA*. Upon insertion into target genes, Tn*phoA* can generate protein fusions between the product of the target gene and AP, the product of *phoA* (14). Such fusion proteins exhibit high AP enzymatic activity only if the portion of the fusion derived from the target gene directs export of the AP moiety to the periplasm (14, 15). In other systems it has been shown that periplasmic or membrane proteins can be fused to AP, resulting in enzymatically active fusion proteins (14, 15). Cytoplasmic proteins, however, cannot yield enzymatically active hybrid proteins when fused to AP (14). Therefore, those regions of DNA in which Pho$^+$ (enzymatically active) Tn*phoA* insertions occur must code for periplasmic or membrane proteins.

Pho$^+$ Tn*phoA* insertions into pKS17 were isolated, and the sites of insertion were determined (Fig. 2B). All such insertions occurred in one region of the chromosomal insert on pKS17. This region overlapped those Tn*phoA* insertions that inactivate *degP*; hence these were fusions of AP to the *degP* gene product. All these Pho$^+$ Tn*phoA* insertions occurred in one orientation, from which we can conclude that the direction of transcription is leftward in Fig. 2. The fusion proteins resulting from six of these insertions were identified by immunoprecipitation with antiserum to AP (Fig. 3). The sizes of these fusion proteins correlated well

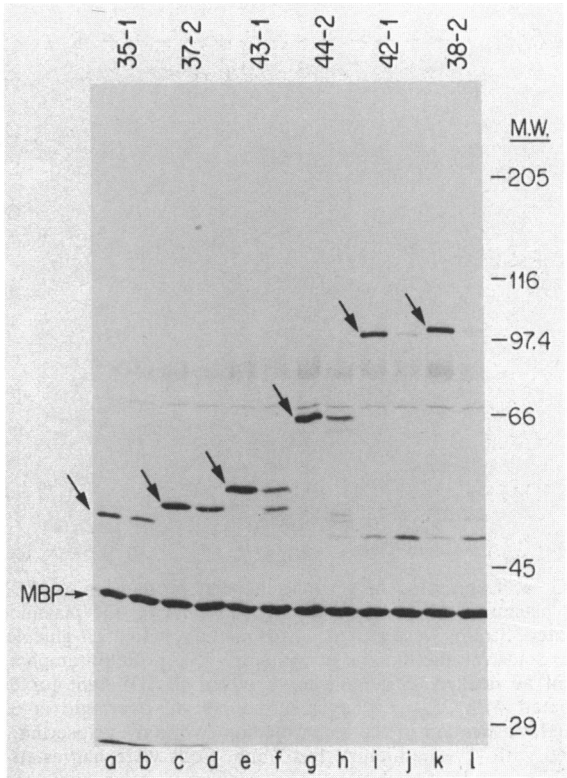

FIG. 3. DegP-AP protein fusions. Strains KS300[pKS17 (*degP*::Tn*phoA*)] were grown in M63 medium–0.2% maltose–18 amino acids (no methionine or cysteine)–25 μg of chloramphenicol per ml at 37°C. Strains were labeled for 2 min with [^{35}S]methionine and harvested by trichloroacetic acid precipitation at 0 min (lanes a, c, e, g, i, and k) or 60 min (lanes b, d, f, h, j, and l) after initiation of pulse-chase with 100 μg of unlabeled methionine per ml. Proteins were precipitated with antisera to AP and maltose-binding protein (MBP). The allele numbers of *degP*::Tn*phoA* insertions are listed above the gel lanes. Arrows point to the positions of DegP-AP fusion proteins.

with the relative positions of Tn*phoA* insertions on pKS17 (Table 4). The largest fusion protein was 97.5 kDa, containing approximately 48 kDa derived from AP and 49.5 kDa derived from the *degP* gene product.

Identification of the plasmid-encoded *degP* gene product. The size of the intact *degP* gene product was determined by expressing plasmid-encoded proteins in maxicells (20). All plasmids which complemented *degP* produced a 50-kDa

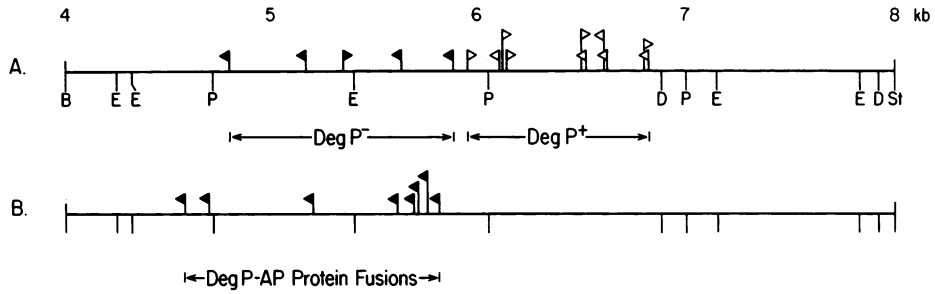

FIG. 2. Tn*phoA* insertion mutations in pKS17 (*degP*). The direction of each flag indicates the orientation of the Tn*phoA* insertion and the direction of transcription of the *phoA* gene. (A) Pho$^-$ insertions. Open flags indicate insertions that complement *degP*, and closed flags indicate insertions that do not complement *degP*. (B) Pho$^+$ insertions. Six of these insertions do not complement *degP*, and two of these insertions do. See text for evidence that all insertions lie in one gene.

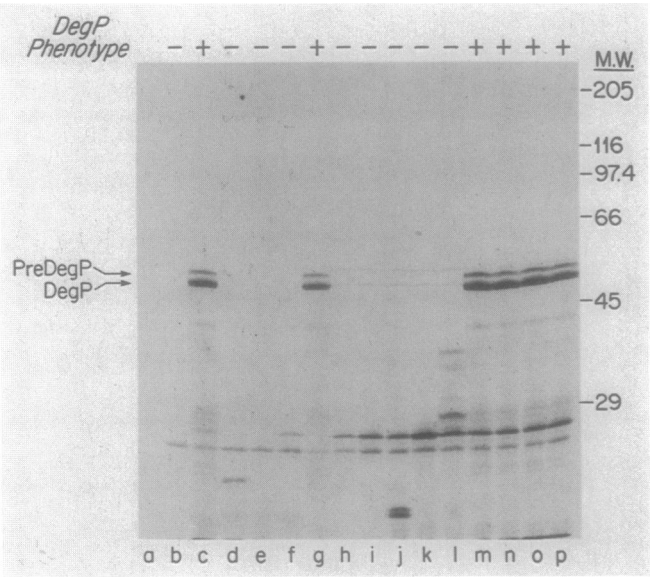

FIG. 4. Expression of plasmid-encoded proteins in *degP*$^+$ and *degP* plasmids. All strains were KS300 carrying the plasmids as indicated. Strains were grown in M63 medium with 0.2% glucose–18 amino acids (no methionine or cysteine)–25 μg of chloramphenicol per ml as needed. Strains were exposed to UV light for 30 s, incubated with 25 μg of cycloserine per ml overnight at 37°C, washed, suspended in the same medium without cycloserine, and labeled with [^{35}S]methionine for 5 min. Cells were harvested and boiled in gel loading buffer. These extracts were subjected to electrophoresis in a 10% sodium dodecyl sulfate-polyacrylamide gel and analyzed by autoradiography. Plasmids in each strain were as follows: lane a, no plasmid; lane b, pACYC184; lane c, pKS12; lane d, pKS14; lane e, pKS15; lane f, pKS16; lane g, pKS17; lane h, pKS17 (*degP10-2*::Tn*phoA*), 5.92 kb; lane i, pKS17 (*degP13-1*::Tn*phoA*), 5.76 kb; lane j, pKS17 (*degP16-1*::Tn*phoA*), 5.48 kb; lane k, pKS17 (*degP18-1*::Tn*phoA*), 5.27 kb; lane l, pKS17 (*degP9-1*::Tn*phoA*), 5.03 kb; lane m, pKS17::Tn*phoA*3-1, 6.13 kb; lane n, pKS17::Tn*phoA*12-3, 5.18 kb; lane o, pKS17::Tn*phoA*10-4, 6.53 kb; and lane p, pKS17::Tn*phoA*13-2, 7.33 kb. The positions in kilobases of Tn*phoA* insertions refer to the restriction map of pKS1 (Fig. 1). Molecular weights (10^3) are indicated.

protein; the same protein was not seen with plasmids that did not complement *degP* (Fig. 4). This 50-kDa protein appears to be the product of the *degP* gene. Another protein slightly larger than this presumptive DegP protein was also expressed only in DegP$^+$ strains (Fig. 4). This second protein may be a precursor to DegP containing a signal sequence.

Phenotypes of *degP*::Tn*phoA* mutations. Tn*phoA* insertions which inactivate the *degP* gene on pKS17 were recombined onto the chromosome (see Materials and Methods) to determine their phenotypes. All resulting strains were shown to contain a Tn*phoA* insertion cotransducible with a Tn*10* insertion 80% linked to *degP*. In the case of Pho$^+$ insertions, we screened for production of AP to obtain the desired recombinants (see Materials and Methods). The resulting strains expressed 5- to 10-fold less AP than the plasmid-bearing strains (Table 4) and contained lower levels of AP fusion proteins (as determined by immunoprecipitation of labeled protein; data not shown). When present in the chromosome, six of the eight Pho$^+$ *degP*::Tn*phoA* mutations conferred the growth defect characteristic of *degP* strains. The other two alleles, *degP42-1*::Tn*phoA* and *degP38-2*::Tn*phoA*, did not confer this growth defect; strains carrying these insertions were phenotypically DegP$^+$. Strains carrying these two *degP* alleles also were capable of degrading Tsr-AP 2 (assayed by pulse-chase labeling with [^{35}S]methionine and immunoprecipitation of labeled proteins as described in reference 21; data not shown). We estimate that the DegP-AP fusion proteins produced in these strains contained approximately 48 and 49.5 kDa of the 50-kDa DegP protein, respectively (Fig. 3). We think it likely that these fusion proteins contain enough of the DegP protein to exhibit DegP function.

In the case of Pho$^-$ insertions, we used screening for failure to grow at high temperature as part of the protocol for identifying the desired recombinants (see Materials and Methods). The resulting strains exhibited the protein degradation defect characteristic of *degP* strains (Table 3).

DISCUSSION

Null mutations in the *degP* gene confer temperature-sensitive growth in many strain backgrounds. This growth phenotype proved quite useful in the mapping, cloning, and characterization of the *degP* gene. *degP* maps at approximately 3.5 min on the *E. coli* chromosome, a region where no previously identified protease genes map. *degP* is thus distinct from other known protease loci. We have also cloned the *degP* gene into pACYC184-derived plasmids. DegP-AP fusions carried on these plasmids are overexpressed approximately 10-fold relative to the chromosomally encoded DegP-AP fusion proteins. Therefore, these plasmids may overproduce the wild-type DegP protein, suggesting that *E. coli* can tolerate elevated levels of DegP. However, this conclusion must be confirmed by chemical or functional assays of the DegP protein itself. DegP expressed from plasmids has an apparent molecular weight of 50,000. Another species of molecular weight 52,000 is also expressed from DegP$^+$ plasmids and may be a precursor of DegP containing a signal sequence. Because DegP-AP fusion proteins have AP enzymatic activity, the DegP protein is located in the cell envelope and the presence of a signal sequence is likely. Resolution of whether DegP protein resides in the periplasm or the inner membrane will require identification of the wild-type DegP protein in cellular fractions prepared from viable cells.

All available evidence is consistent with *degP* coding for a protease. The DegP gene product is located in the cell envelope, where proteins stabilized by *degP* mutations are located and hence where the protease must be located. DegP-AP fusion proteins 42-1 and 38-2 provide additional evidence that *degP* encodes a protease. These DegP-AP fusion proteins, which possess DegP activity, are extremely unstable. After a 2-minute labeling in vivo, one can detect AP-sized breakdown products of these proteins, yet other fusion proteins do not show any such breakdown products until later (Fig. 3). The extremely rapid breakdown of DegP-AP 42-1 and 38-2 may indicate intramolecular proteolysis of the proteins by the active DegP moiety. However, one cannot firmly conclude that the DegP protein is a protease until direct evidence is obtained.

Our results strengthen the suggestion that *degP* plays an important role in cell physiology. All *degP* alleles characterized confer two phenotypes: reduced proteolysis of cell envelope proteins and failure to grow at high temperature. Because all *degP* mutations tested are either deletions or insertions, which can exert extreme polar effects in an operon, it seemed possible that both phenotypes were caused not by absence of the 50,000-molecular-weight DegP protein but by polarity effects. One or both phenotypes

might be due to absence of some other protein encoded distally in an operon. The phenotypes of *degP*::Tn*phoA* insertions 42-1 and 38-2 argue that both phenotypes are indeed caused by absence of the DegP protein. These two insertions are phenotypically DegP$^+$ (able to degrade periplasmic proteins and to grow at high temperature). The Tn*phoA* insertions should be polar on any distal genes in an operon, so it appears that any polar effects are not responsible for either the protein degradation or growth phenotype. Therefore, we conclude that it is only the absence of the 50,000-molecular-weight protein which causes *degP* strains to exhibit both phenotypes.

Although *lpp*$^+$ *degP* strains are unable to grow at 42°C, isogenic *lpp degP* strains are viable under identical conditions. Because *lpp* mutants release periplasmic proteins to the medium (22), the difference in viability between these strains raises the possibility that *degP lpp*$^+$ strains accumulate some toxic substance in the periplasm (e.g., some toxic polypeptide normally degraded by DegP). In the *lpp-5508* strain background this toxic substance can at least partially be released from the cell and thereby be reduced to a level that does not prevent growth.

We also showed that expression of unstable or stable fusion proteins at high levels can cause *degP lpp* strains to be inviable at high temperature. Since stable proteins accumulate in both *degP*$^+$ and *degP* strains but are toxic only to *degP* strains, the stable proteins are not directly toxic but rather must inhibit growth by their ability to potentiate some physiological abnormality present in *degP* strains (such as the toxicity of the growth-inhibitory polypeptide postulated above). All situations which inhibit the growth of *degP lpp* strains share the property of causing increased expression of proteins in the periplasm or inner membrane. One explanation for these phenomena is that abnormal proteins (or abnormally high levels of maltose transport proteins) increase the concentration of the inhibitory substance which accumulates in the periplasmic space of *degP* strains. For instance, an increase in the protein content of the periplasm may interfere with leakage of the toxic substance through the outer membrane. Another possibility is that the toxic compound is slowly degraded in *degP* strains and that abnormal proteins inhibit the enzyme responsible for this residual degradation, thereby increasing the steady-state level of the toxic material. Further studies of the growth defect of *degP* strains will allow us to test this model and may identify the toxic polypeptide and the mechanism of its toxicity. These arguments to explain the growth properties of *degP* strains are highly speculative, but they do provide some context in which to relate the growth defect to reduced proteolysis, which we believe is the primary defect of *degP* mutants.

The temperature dependence of this growth defect suggests that DegP function is most important to the cell at elevated growth temperatures. This is consistent with observations that the total rate of protein turnover increases at elevated growth temperatures (19) and that cytoplasmic proteolysis is under control of the heat shock response (2). Recently, we learned that other workers have independently identified the *degP* locus in a search for heat-shock-related functions. The *htrA* gene described by Lipinska et al. (11a) is the same as *degP*.

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