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Oligopeptidase A Is Required for Normal Phage P22 Development

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The *opdA* gene of *Salmonella typhimurium* encodes an endoprotease, oligopeptidase A (OpdA). Strains carrying *opdA* mutations were deficient as hosts for phage P22. P22 and the closely related phages L and A3 formed tiny plaques on an *opdA* host. *Salmonella* phages 9NA, KB1, and ES18.h1 were not affected by *opdA* mutations. Although *opdA* strains displayed normal doubling times and were infected by P22 as efficiently as *opdA*$^+$ strains, the burst size of infectious particles from an *opdA* host was less than 1/10 of that from an *opdA*$^+$ host. This decrease resulted from a reduced efficiency of plating of particles from an *opdA* infection. In the absence of a functional *opdA* gene, most of the P22 particles are defective. To identify the target of OpdA action, P22 mutants which formed plaques larger than wild-type plaques on an *opdA* mutant lawn were isolated. Marker rescue experiments using cloned fragments of P22 DNA localized these mutations to a 1-kb fragment. The nucleotide sequence of this fragment and a contiguous region (including all of both P22 gene *7* and gene *14*) was determined. The mutations leading to *opdA* independence affected the region of gene *7* coding for the amino terminus of gp7, a protein required for DNA injection by the phage. Comparison of the nucleotide sequence with the N-terminal amino acid sequence of gp7 suggested that a 20-amino-acid peptide is removed from gp7 during phage development. Further experiments showed that this processing was *opdA* dependent and rapid (half-life, <2 min) and occurred in the absence of other phage proteins. The *opdA*-independent mutations lead to mutant forms of gp7 which function without processing.

Oligopeptidase A (OpdA) was identified by Vimr and Miller (42) as one of two enzymes in extracts of *Salmonella typhimurium* which hydrolyzed *N*-acetyl-L-Ala_4. OpdA has been purified from both *Escherichia coli* (28) and *S. typhimurium* (9). Novak et al. (28) and Novak and Dev (27) have shown that OpdA is a signal peptide peptidase. In vitro, OpdA is the major soluble enzyme able to hydrolyze the *E. coli* lipoprotein signal peptide. Substrate specificity studies indicate that OpdA is an endopeptidase with a preference for Ala and Gly residues (27, 28, 41). Its inhibition by metal chelators (9, 28) suggests that it is a metallopeptidase.

The gene encoding OpdA, *opdA*, has been cloned and sequenced (9). The predicted amino acid sequence of the 77-kDa OpdA protein contains a thermolysin-type metalloprotease Zn^{2+}-binding site (9, 22). The amino acid sequence shows strong similarity to another bacterial peptidase (dipeptidyl carboxypeptidase, encoded by *dcp*) and to a mammalian metalloprotease (rat metallopeptidase EP 24.15) that is involved in the processing of peptide hormones (9). Evidence presented in the accompanying article shows that *opdA* is the *Salmonella* homolog of the *E. coli prlC* gene (10). Mutant alleles of *prlC* suppress the localization defect of certain signal peptide mutations (39).

Mutations in *opdA* were originally isolated by screening for mutants unable to use $AcAla_4$ as a sole nitrogen source. During the characterization of these *opdA* mutations, it was noticed that phage P22 made much smaller plaques on an *opdA* host than on an *opdA*$^+$ host (40). This was somewhat surprising because P22, in contrast to many other related phages, is not known to require proteolytic processing steps in its development (2, 6). In the hope of gaining insight into the role of *opdA* in cellular processes, we have begun a study of its function in the development of phage P22. This article describes experiments aimed at characterizing the P22 defect conferred by *opdA* mutant hosts and identifying the phage target of *opdA* action.

MATERIALS AND METHODS

Bacterial and phage strains. All *S. typhimurium* strains used were derived from LT2 and are listed in Table 1. *E. coli* DH5 (16) was used to propagate plasmids for sequencing. Phage strains used and their sources are also listed in Table 1. Plasmid vectors used included pBR322 (1), pBR328 (37), pSE380 (3), and pT7-5, a derivative of pT7-1 (38). The construction of other plasmids used is described in Table 1. Bacteria were routinely grown in LB medium (32) at 37°C. LB medium with galactose (0.05%) was used to propagate phage P22 on *galE* strains. E medium (43) was used as the minimal medium and supplemented with appropriate amino acids at 0.4 mM and glucose at 4 g/liter. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml in liquid media and 50 μg/ml in solid media; chloramphenicol, 25 μg/ml; tetracycline, 25 μg/ml; and kanamycin, 50 μg/ml.

Phage propagation and purification of phage particles. Small-scale lysates of P22 phage were made essentially as described by Davis et al. (11). Large-scale purification of phage was performed essentially as described by Sambrook et al. (32). Host strains were grown in liquid culture to an optical density at 600 nm (OD_{600}) of 0.5, infected with phage at a multiplicity of infection of 1, and incubated for about 3 h before the addition of $CHCl_3$ (2%, vol/vol). DNase and RNase were added to a final concentration of 1 μg of each

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TABLE 1. Bacterial and phage strains and plasmids used in this study

Strain or plasmid	Genotype, description, source, or reference[a]
S. typhimurium	
TN1292	*dcp-1 zci-845*::Tn*10 zhg-848*::Tn*5*
TN1293	*dcp-1 zci-845*::Tn*10 opdA1 zhg-848*::Tn*5*
TN1379	*leuBCD485*
TN2684	*lon-101 zba-883*::Tn*10*
TN3101	*leuBCD485 opdA10*::MudJ
TN3491	TN3101/pCM172
TN3625	TN1379/pCM187
TN3627	TN3101/pCM187
TN3629	TN1379/pCM185
TN3630	TN3101/pCM185
TN3647	*leuBCD485 trp*::[Φ(*placUV5*-T7 gene *1*) *lacI*^{q} Spc^r][b]
TN3684	TN3647/pT7-5
TN3685	TN3647/pCM193
TN3803	TN3101/pDH3 (*mutD5*)
TN3804	*purC7 recA1 srl-202*::Tn*10*/F'42 *finP301*/pCM172
TN3428	*hsdSA29 hsdSB121 hsdL6 metA22 metE551 trpC2 ilv-452 fla-66 rpsL120 xyl-404 galE719 nml H1-b H2-e,n,x* (cured of Fels2) (=JR501 [31])
DB7155	*leuA414*(Am) *hisC527*(Am) *supE20* (44)
Phages	
9NA	B. A. D. Stocker
A3	B. A. D. Stocker
ES18.h1	B. A. D. Stocker
L	B. A. D. Stocker
KB1	B. A. D. Stocker
P22 *c2*H5	
P22 HT12/4 *int*103	J. R. Roth
P22 *c2*H5 *13am*H101	This study
P22 *14am*H600 *c2-5 sieA444*	MS523 (M. M. Susskind)
P22 *14am*H611 *c2-5 sieA444*	MS679 (M. M. Susskind)
P22 *7am*H1035 *c1-7 h21*	MS1029 (M. M. Susskind)
P22 *7am*H1375 *c1-7*	A. R. Poteete
P22 *20am*H1030 *c1-7 h21*	MS1212 (M. M. Susskind)
P22 7-1a *c2*H5	P22 1a (this study)
P22 7-3a *c2*H5	P22 3a (this study)
P22 7-4a *c2*H5	P22 4a (this study)
Plasmids	
pDH3	*mutD5* on pBR322 (R. A. Maurer)
pCM146	9.2-kb *Eco*RI fragment of P22 4a in *Eco*RI site of pBR328
pCM147	5.6-kb *Eco*RI-*Sal*I fragment of P22 4a in *Eco*RI-*Sal*I sites of pBR328
pCM148	3.2-kb *Eco*RV-*Sal*I fragment of P22 4a in *Sal*I -*Pvu*II sites of pBR328
pCM149	1.5-kb *Eco*RV-*Ava*I fragment of P22 4a in *Ava*I-*Pvu*II sites of pBR328
pCM150	1-kb *Ava*I (*Bam*HI linker)-*Bst*EII (*Bam*HI linker) fragment of P22 4a in *Bam*HI site of pBR322
pCM151	0.5-kb *Eco*RV-*Bst*EII (blunt) fragment of P22 4a in *Ava*I (blunt)-*Pvu*II sites of pBR328
pCM152	1.7-kb *Sal*I-*Hpa*I fragment of P22 4a in *Sal*I-*Pvu*II sites of pBR328
pCM153	3.4-kb *Eco*RI-*Sal*I fragment of P22 4a in *Sal*I-*Eco*RI sites of pBR328
pCM158	3-kb *Sal*I-*Eco*RV (*Hin*dIII linker) fragment of P22 4a in *Sal*I-*Hin*dIII sites of pSE380
pCM166	1.5-kb *Eco*RV-*Ava*I fragment of P22 1a in *Ava*I-*Pvu*II sites of pBR328
pCM167	1.5-kb *Eco*RV-*Ava*I fragment of P22 3a in *Ava*I-*Pvu*II sites of pBR328
pCM168	1.5-kb *Eco*RV-*Ava*I fragment of P22 *c2*H5 in *Ava*I-*Pvu*II sites of pBR328
pCM172	2.8-kb *Eco*RV (*Hin*dIII linker)-*Cla*I (blunt) fragment of P22 4a in *Nco*I (blunt)-*Hin*dIII sites of pSE380
pCM179	1-kb *Ava*I (*Bam*HI linker)-*Bst*EII (*Bam*HI linker) fragment of P22 *c2*H5 in *Bam*HI site of pBR322
pCM185	1.5-kb *Eco*RV (*Hin*dIII linker)-*Ava*I (blunt) fragment of P22 4a in *Nco*I (blunt)-*Hin*dIII sites of pSE380
pCM186	pSE380 *Nco*I (blunt), religated
pCM187	1-kb *Ava*I (*Bam*HI linker)-*Bst*EII (*Bam*HI linker) fragment of P22 *c2*H5 in *Bam*HI site of pCM186, correct orientation for transcription of gene *7* from *trc* promoter
pCM188	1-kb *Ava*I (*Bam*HI linker)-*Bst*EII (*Bam*HI linker) fragment of P22 *c2*H5 in *Bam*HI site of pCM186, orientation opposite to that of pCM187
pCM193	*Eco*RI-*Hin*dIII fragment from pCM187 in *Eco*RI-*Hin*dIII sites of pT7-5

[a] Sites designated blunt were first cleaved with the indicated restriction enzyme, and then ends were made flush by treatment with S1 nuclease and the Klenow fragment of DNA polymerase I (32). Sites with linkers were first cleaved with the indicated restriction enzyme and made blunt if necessary, and then short oligonucleotides containing the indicated restriction site (New England Biolabs) were ligated.

[b] The construction of this strain by J. L. Miller will be described elsewhere (26a).

per ml, and the suspension was incubated at room temperature for 30 min. NaCl was then added to 1 M, and the suspension was incubated on ice for 1 h. After removal of cell debris by centrifugation (11,000 × *g*, 10 min, 4°C), phage was precipitated by the addition of polyethylene glycol 8000 (final concentration, 10%). Phage was allowed to precipitate overnight at 4°C, pelleted at 11,000 × *g* for 15 min, and resuspended in 10 ml of TM (50 mM Tris-HCl [pH 7.8], 10 mM $MgCl_2$). For *13am* phage, cells were infected at a multiplicity of infection of ~10. Infected cells were incubated for 2 h and then pelleted by centrifugation, resuspended in 0.01 volume of TM, and lysed by the addition of $CHCl_3$. Cell debris was removed from this concentrated phage suspension by centrifugation (11,000 × *g*, 15 min). Phage was further purified by isopycnic CsCl gradient centrifugation as follows. Solid CsCl was added to the phage suspension (0.75 g/ml of original suspension), which was then centrifuged in a 70Ti rotor (150,000 × *g*, 4°C, 24 to 48 h). The opalescent band of phage particles was removed by puncturing the side of the centrifuge tube. CsCl was removed by passing the phage suspension over a small Sephadex G-10 column (PD10; Pharmacia) previously equilibrated with TM. The titer of the resulting suspension was determined by plating on TN1379 (*sup*^{0}), DB7155 (*sup*$^+$), or TN3101 (*opdA*), and the efficiency of plating (EOP; PFU/OD_{260}) was determined.

Isolation of phage mutants. P22 mutants which made large plaques on *opdA* hosts were isolated as follows. An overnight culture of strain TN3803 [*opdA10*::Mu*dJ*/pDH3 (*mutD5*)] was diluted 1:100 into 50 ml of LB-ampicillin medium and incubated with shaking at 37°C for 2 h. P22 *c2*H5 (a clear-plaque mutant) (10^8 PFU) was added, and the cultures were incubated overnight. $CHCl_3$ (0.5 ml) was added, and the debris was pelleted. The titer of the supernatant was determined on TN3101 (*opdA10*::Mu*dJ*). Large plaques (about 1 in 5,000) were picked and repurified twice on a lawn of TN3101. Single plaques were used to make lysates on TN3101 which were stored at 4°C over $CHCl_3$. Only one mutant was saved from each original lysate.

Subcloning phage DNA. To purify phage DNA, phage particles were concentrated by centrifugation (30,000 × *g*, 90 min, 4°C), and resuspended in 1 ml of TM. This suspension was extracted with equal volumes of phenol (equilibrated with Tris-HCl [pH 7.8]) and then repeatedly extracted with equal volumes of phenol-$CHCl_3$-isoamyl alcohol (25:24:1, vol/vol/vol) until no precipitate was seen at the interface. After a final extraction with $CHCl_3$-isoamyl alcohol (24:1), the aqueous layer was removed, and the DNA was precipitated at −20°C by the addition of 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol, pelleted at 12,000 × *g* for 30 min, and washed with 70% ethanol (4°C). P22 DNA was digested with restriction endonucleases (Bethesda Research Laboratories) according to the manufacturer's recommendations. DNA fragments were purified with Gene Clean (Bio 101, Inc.), according to the manufacturer's directions, after being separated on agarose gels. Fragments were ligated to cleaved plasmid vectors by standard protocols (32).

Marker rescue by plasmids containing P22 DNA. To detect marker rescue by plasmids containing mutant phage DNA, strain TN3428 containing the recombinant plasmid to be tested was grown overnight in LB-galactose medium. A small-scale lysate was made on this host by using P22 *c2*H5. The titer of this phage lysate was determined on both TN3101 (*opdA*) and TN1379 (*opdA*$^+$). The number of large (recombinant) plaques on TN3101 divided by the number of plaques on TN1379 determined the frequency of recombination. Marker rescue of amber mutations by plasmids containing phage DNA was performed by moving the plasmid into the *supE* strain DB7155. This permissive host was infected with the mutant phage to be tested, and the resulting lysate was titered on both DB7155 (*supE*) and TN1379 (*sup*^{0}).

Dominance testing. The ability of gene *7* mutations to allow *7*$^+$ P22 to form large plaques on an *opdA* lawn was tested as follows. An overnight culture of TN3491 (*opdA10*::Mu*dJ*/pCM172) was diluted 1:100 into LB medium and incubated with shaking for 1 to 2 h. The culture was made 1 mM in isopropyl-β-D-thiogalactopyranoside (IPTG) to induce the expression of the mutant gene *7* from the *trc* promoter. After incubation for 1 h, 0.5 ml of this culture was combined with P22 *c2*H5 (200 PFU) and 2.5 ml of LB soft agar (0.75% agar) and plated on LB agar containing ampicillin and IPTG (1 mM). These plates were incubated overnight and then examined for large plaques. Transposon Tn*1000* insertions which eliminated the ability of pCM172 to restore the normal plaque size were isolated by the method of Guyer (15). TN3804 (*recA1*/F'42 *finP301*/pCM172) was mixed with TN3101, and transconjugants were selected on LB-ampicillin-kanamycin medium. Transconjugants were purified and tested for their ability to allow development of normal large plaques. Of the 32 transconjugants tested, 5 displayed only tiny plaques when infected with P22 *c2*H5. The locations of these five Tn*1000* insertions were determined by restriction mapping and later confirmed by nucleotide sequencing.

Visualization of P22 proteins. Pulse-chase experiments were performed to visualize the proteins expressed from pSE380-based plasmids. An overnight culture of the plasmid-containing strain was diluted 1:50 into minimal glucose medium with leucine and ampicillin. The culture was incubated at 37°C until it reached an OD_{600} of 0.8, and then IPTG was added to 1 mM. After further incubation to allow induction of the *trc* promoter, a mixture of ^{14}C-amino acids (55.5 mCi per milliatom of carbon) was added to a final concentration of 10 or 30 μCi/ml. After either 1 or 5 min, Casamino Acids were added to 1%. Samples (0.2 ml) were taken at times after the addition of Casamino Acids and put on ice. Cells were pelleted, resuspended in 20 or 50 μl of sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol), and heated to 95°C for 3 to 5 min. If necessary, samples were briefly sonicated to reduce viscosity before being loaded onto SDS-polyacrylamide gels for electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) was performed with 12% polyacrylamide slab gels and the Tris-glycine buffer system of Laemmli (24). After electrophoresis, the gels were stained with Coomassie blue, destained, and dried. Bands of radiolabelled protein were detected by exposure to Kodak X-Omat AR film.

Cloned gp7 was expressed from a phage T7 promoter as follows. An overnight culture of TN3685(pCM193) was diluted 1:50 into minimal glucose medium with leucine, tryptophan, and ampicillin, and incubated to an OD_{600} of 0.5. Expression of the T7 RNA polymerase was induced by adding IPTG to 1 mM. After 30 min at 37°C, rifampin (20 mg/ml in dimethyl sulfoxide) was added to 0.4 mg/ml, and after 30 min of additional incubation at 37°C, mixed ^{14}C-amino acids (55.5 mCi per milliatom of carbon) (NEN Research Products, E. I. du Pont de Nemours & Company) were added to 3 μCi/ml. After 1 min, Casamino Acids were added to a 1% final concentration. Samples were removed and placed on ice at various times after the pulse of radio-

activity, pelleted, resuspended in 50 μl of SDS sample buffer, boiled for 3 min, and electrophoresed in 10% Tris–Tricine–polyacrylamide gels (34). After being stained with Coomassie blue, the gels were dried, and radiolabelled proteins were detected by autoradiography.

DNA sequencing. All DNA sequencing was performed by using the dideoxynucleotide chain termination method (33) and Sequenase (United States Biochemical). Double-stranded plasmid templates were prepared as described by Chen and Seeburg (7). The sequence was obtained by using primers to the vector (New England Biolabs), primers to the ends of the Tn*1000* insertions in pCM172 (25), and other oligonucleotide primers synthesized specifically for this project. The sequence of gene *14* from mutant phage strains MS523 (*14am*H600) and MS679 (*14am*H611) was obtained by using a template amplified by the polymerase chain reaction as previously described (26).

Nucleotide sequence accession number. The region of P22 sequenced, including genes *7* and *14*, has been assigned GenBank accession number M93985.

RESULTS

***opdA* mutations affect phage P22 development.** During the characterization of *S. typhimurium opdA* mutations, it was noticed that bacteriophage P22 made pinpoint plaques on an *opdA* host. Two independent *opdA* mutations, *opdA1* and *opdA2*, had the same effect on P22 plaque size. Strains carrying *opdA3*, a mutation which leads to levels of peptidase activity intermediate between those of the wild type and *opdA1* mutants (41), yielded plaques which were intermediate in size between those on *opdA1* and *opdA*$^+$ hosts. To determine whether the *opdA* small-plaque phenotype was specific for phage P22, phages P22, A3, L, KB1, 9NA, and ES18.h1 were plated on lawns of *opdA1* (TN1293) and *opdA*$^+$ (TN1292) strains. Only P22 and the closely related phages L and A3 made smaller plaques on an *opdA* host than on an *opdA*$^+$ host. Furthermore, the small plaques are not simply the result of slow growth by *opdA* strains; *opdA*$^+$ (TN1379) and *opdA* (TN3101) strains had the same generation times in LB medium (24 min at 37°C). Thus, the small P22 plaques are the result of a specific effect on the growth of P22 and closely related phages.

The tiny plaques seen on an *opdA* mutant lawn were not the result of a defect in the ability of *opdA* strains to be infected by phage P22. P22 *c2*H5 was able to infect an *opdA* host with the same efficiency as it did an *opdA*$^+$ host, as determined by the number of $CHCl_3$-sensitive infectious centers present after incubation of phage and host cells. Similarly, the number of transductants obtained by transducing an *opdA* strain with P22 HT12/4 *int*103 transducing lysates prepared on an *opdA*$^+$ host was the same as that obtained by transducing an *opdA*$^+$ recipient.

The following results demonstrate that the yield of infectious particles from infection of an *opdA* host is less than that from an *opdA*$^+$ host. When *opdA* mutations were moved by P22 transduction, it was noticed that the titers of transducing lysates made on *opdA* strains were much lower than those typically obtained from a lysate made on an *opdA*$^+$ strain. This effect was quantitated by measuring the yield of infectious particles from single bursts of phage after infection by P22 *c2*H5 of the *opdA* host, TN3101, and the *opdA*$^+$ host, TN1379. Infection of TN3101 resulted in a burst of phage that was only 5 to 10% of that of TN1379 (38 and 450 PFU per infectious center, respectively).

The diminished yield of infectious particles from an *opdA* strain was not simply the result of fewer phage particles being produced; rather, the particles produced from an *opdA* strain are defective. Phage particles produced after infection of *opdA*$^+$ (TN1379) and *opdA* (TN3101) hosts were purified by isopycnic CsCl gradient centrifugation. The EOP of phage grown on TN3101, 2.3×10^{10} PFU/OD_{260} unit, was about 5% of that of phage grown on TN1379, 6.5×10^{11} PFU/OD_{260} unit. In other words, about 95% of the phage produced from an *opdA* infection was unable to form plaques. Thus, in an *opdA* host, there is a reduced yield of infectious phage.

Isolation of *opdA*-independent mutants. To define the P22 function which requires *opdA* for full activity, phage mutations which led to larger plaques on an *opdA* host were sought. To enrich for such mutants, P22 *c2*H5 lysates were made on TN3101 containing plasmid pDH3. TN3101 contains a nonreverting *opdA10*::Mu*dJ* insertion in *opdA*, and pDH3 contains the *E. coli mutD5* allele to enhance mutation frequency. Three independent phage mutants which made larger plaques on TN3101 than did P22 *c2*H5 were isolated. Because *S. typhimurium* LT2, from which the host strain was derived, has been shown to release several different kinds of phage following P22 infection (46), we confirmed that the *opdA*-independent mutant phages were indeed P22. First, the mutant phages made clear plaques, as did the parent phage, P22 *c2*H5. Second, the fragments generated when DNA from these phages was digested with three endonucleases, *Bam*HI, *Eco*RI, and *Hin*dIII, were the same sizes as those from P22 *c2*H5 (data not shown). Thus, we were confident that these mutant phages were P22.

Figure 1 shows plaques made by the mutant phages on lawns of *opdA*$^+$ and *opdA* bacteria. Although all three P22 mutants made larger plaques on an *opdA* mutant lawn than did wild-type P22 *c2*H5, the plaque phenotypes of the three mutants were different. Of the three, mutant 4a made the largest plaques on an *opdA* host lawn. Plaques made by mutant 4a on an *opdA*$^+$ host lawn were similar in size to those made on an *opdA* host lawn. Plaques made by mutant 3a on an *opdA*$^+$ host lawn were slightly smaller than those on an *opdA* host lawn. Plaques of mutant 1a on an *opdA* host lawn were significantly smaller than on an *opdA*$^+$ host lawn, although still much larger than those made by the parent. Mutant phages were purified from lysates of TN1379 (*opdA*$^+$) and TN3101 (*opdA*) by isopycnic CsCl gradient centrifugation, and the EOP was measured for each mutant on an *opdA*$^+$ host (TN1379). The relative EOPs (EOP on an *opdA* host/EOP on an *opdA*$^+$ host) of P22 *c2*H5 and the mutants were as follows: P22 *c2*H5, 0.05; mutant 4a, 1.3; mutant 3a, 0.91; and mutant 1a, 0.27. These results are generally consistent with the plaque sizes. They also demonstrate that the mutations in these phages allow them to produce a higher proportion of plaque-forming particles in the absence of *opdA* than the parent phage P22 *c2*H5 produces. Furthermore, they confirm that these mutants are different from each other.

Mapping phage mutations. Marker rescue was used to identify the P22 gene or genes carrying the *opdA*-independent mutations. Restriction fragments of DNA from the mutant phages were subcloned into pBR322 or pBR328. P22 *c2*H5 lysates were prepared on permissive *opdA*$^+$ strains carrying these plasmids, and the presence of recombinant phage in these lysates was detected by plating them on a lawn of an *opdA* host. The presence of large plaques indicated that the fragment of P22 DNA in the plasmid carried the mutation. Initially, a 9.2-kb *Eco*RI fragment of P22 mutant 4a DNA, which contained most of the P22 genes

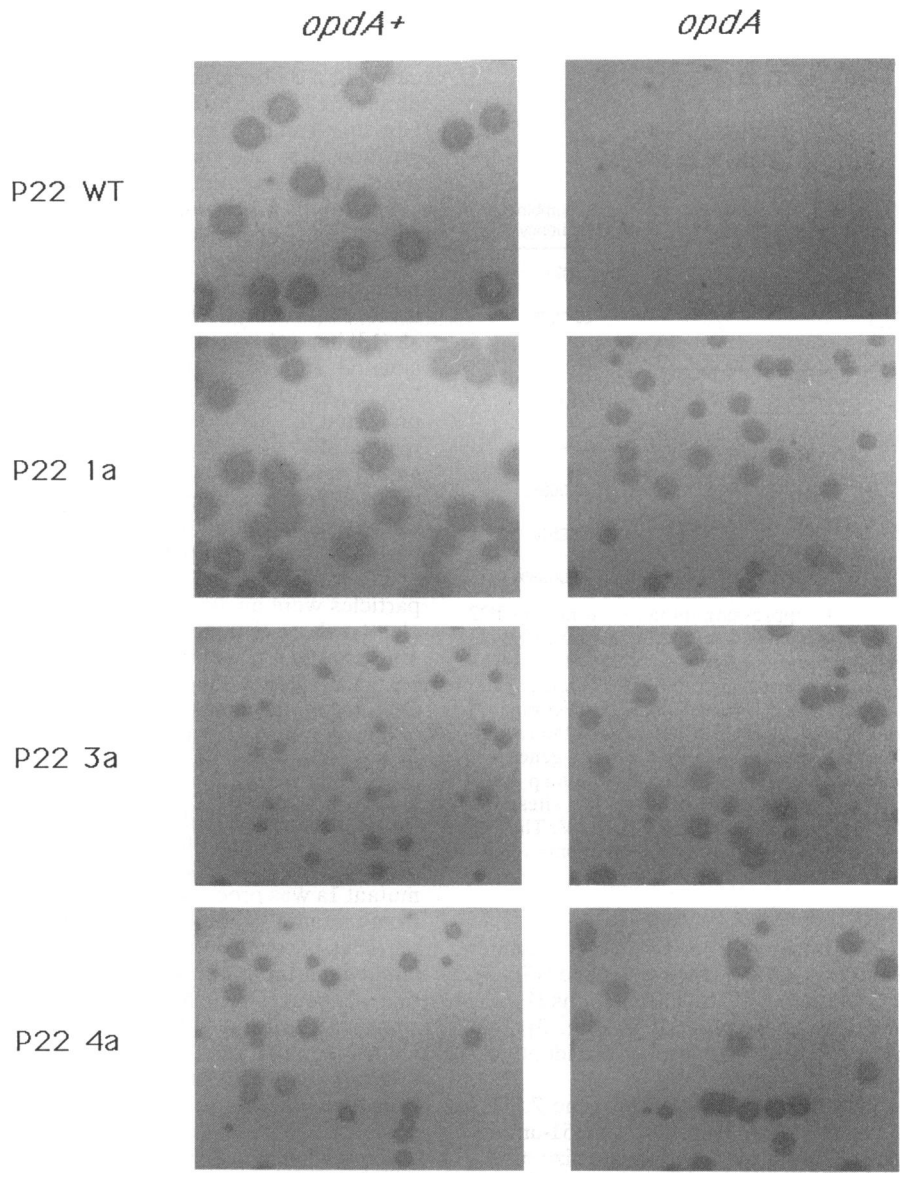

FIG. 1. P22 plaques on *opdA*$^+$ and *opdA* hosts. The host strains were TN1379 (*opdA*$^+$) and TN3101 (*opdA10*::MudJ). WT, wild type.

coding for proteins present in the capsid (genes *8*, *5*, *4*, *10*, *26*, *14*, *7*, *20*, and *16*) (5, 8), was cloned and found to contain the mutation. Progressively smaller fragments were screened until a 1-kb *Ava*I-*Bst*EII fragment containing mutation 4a was identified (pCM150) (Fig. 2). Mutations from mutants 1a and 3a were mapped somewhat less precisely to a slightly larger (1.5-kb) *Ava*I-*Eco*RV fragment which included the 1-kb *Ava*I-*Bst*EII fragment (pCM166 and pCM167, respectively). Both the *Ava*I-*Eco*RV fragment and the *Ava*I-*Bst*EII fragment from P22 *c2*H5 were also cloned, resulting in plasmids pCM168 and pCM179, respectively. The published restriction maps of P22 (5, 8) suggested that the *Ava*I-*Bst*EII fragment contained sequences from gene *14*, *7*, or *20*. To identify the genes present on this fragment, the ability of pCM179 to rescue P22 strains with amber mutations in genes *14*, *7*, and *20* was tested. Plasmid pCM179 was able to rescue gene *7am*H1035 but not *20am*H1030 or *14am*H600. A 1.5-kb *Ava*I-*Eco*RV fragment in pCM168, which extended 500 bp 3′ of the fragment in pCM179, was able to rescue *20am*H1030. These results showed that the 1-kb *Ava*I-*Bst*EII fragment in pCM179 contained the region of gene *7* which included the amber mutation H1035 but did not contain the regions of genes *14* and *20* represented by mutations H600 and H1030, respectively.

Sequence of P22 genes *7* and *14*. The region of phage P22 containing the *opdA*-independent mutations had not previously been sequenced. In order to characterize these mutations further, we determined the nucleotide sequence of a 1.5-kb region including the 1-kb *Ava*I-*Bst*EII fragment and an additional 566 bp 5′ from the *Ava*I site. The nucleotide sequence and the translations of predicted open reading frames (ORFs) are shown in Fig. 3. The *Ava*I-*Bst*EII region contained an ORF (nucleotides 611 to 1297) encoding 229 amino acids. The molecular weight (MW) of the protein predicted by this ORF (23,405) suggests that it encodes gp7 (MW, 22,000) (45), not gp14 (MW, 15,500) (45). To confirm

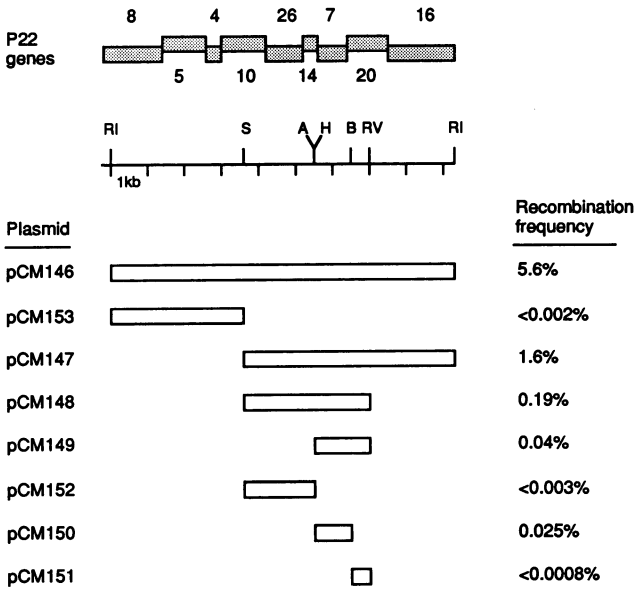

FIG. 2. Mapping the *opdA* suppressing mutation in mutant P22 4a by marker rescue. P22 *c2*H5 lysates were made on strain TN3428 containing plasmids carrying various regions of P22 4a DNA. The titers of these lysates were determined on both TN1379 (*opdA*$^+$) and TN3101 (*opdA*). The number of large plaques on TN3101 compared with the total number of plaques on TN1379 determined the recombination frequency. The approximate positions of P22 genes are indicated by the shaded bars. Below them is a restriction map of the relevant region of the P22 genome (5, 8). Restriction sites: RI, *Eco*RI; S, *Sal*I; A, *Ava*I; H, *Hpa*I; B, *Bst*EII; RV, *Eco*RV. The P22 DNA carried on each plasmid is indicated by the open bars.

this assignment, a 1-kb fragment from two gene *7* amber mutants, H1035 (29) and H1375 (45), was sequenced (Fig. 3). Both mutations were identified as C-to-T transitions (H1035 at position 1019 and H1375 at position 1073 [Fig. 3]) that created UAG codons in the ORF, confirming the identification of the ORF as gene *7*.

Another ORF was identified upstream from gene *7*. This ORF (nucleotides 153 to 605 [Fig. 3]) predicts a 151-amino-acid protein (MW, 17,240). Both the location and size of this ORF suggest that it is gene *14*, which is known to encode gp14, a 15.5-kDa protein (45). This assignment was confirmed by sequencing a 500-bp fragment from two independent gene *14* amber mutants (H600 and H611) (45). Both of these fragments carried the same mutation, a C-to-T transition at position 435 which changed a CAG (Gln) codon in the ORF to UAG, confirming that this ORF is gene *14*. These results also confirmed the marker rescue results (*14am*H600 was not rescued by the 1-kb *Ava*I-*Bst*EII fragment). The sequence 3′ to gene *7* contained the start of an ORF expected to be that of gene *20*. The product of gene *20*, gp20, is a 43.5-kDa protein (45), so pCM179 carries only the N-terminal third of gene *20*.

Sequence of *opdA*-independent mutations. The sequence results show that pCM150 carries only about 30 bp of gene *14*, about 250 bp of gene *20*, and all of gene *7*. We guessed that gene *7* was likely to be the target for mutations that allow *opdA* independence. To test this hypothesis, the gene *7* regions of the three *opdA* independent mutants were sequenced (Fig. 4). Mutants 1a and 4a were each found to carry two mutations which affected the gene *7* ORF. Mutant 1a carried C627A (Thr-6→Lys) and A651G (Glu-14→Gly), while mutant 4a carried G653A (Glu-15→Lys) and G668A (Glu-20→Lys). Mutant 3a was also a multisite mutation, carrying a 39-bp in-frame deletion (nucleotides 650 to 688) predicted to lead to deletion of 13 amino acids (Glu-14 to Gly-26). Mutant 3a also had a silent T-to-C transition at position 826. We conclude that mutations in gene *7* can overcome the requirement for the host gene product OpdA.

Proteolytic processing of gp7. Identification of gene *7* as the site of *opdA*-independent mutations suggested that gp7 might be the direct target of OpdA action. The most obvious mechanism by which OpdA, a protease, could act on gp7 involves proteolytic processing of gp7 by OpdA. Eppler et al. (13) have obtained N-terminal sequence of the gp7 protein isolated from P22 particles. This sequence, ?GG?GGA DKSA, is not found at the N terminus of the gene *7* ORF predicted by our sequence but is found instead beginning at residue 21: KGGKGGADKSA. This suggested that 20 amino acids are removed from the N terminus to generate the gp7 found in phage particles. To determine whether OpdA was required for this processing, P22 *c2*H5 *13am* particles were prepared from *opdA*$^+$ (TN1379) and *opdA10*::Mu*dJ* (TN3101) hosts, and the proteins in the particles were analyzed by SDS-PAGE. The results (Fig. 5) clearly show that gp7 from phage prepared on the *opdA* mutant host is about 2 kDa larger than that from the *opdA*$^+$ particles. This is the predicted size difference between the ORF beginning at position 611 and that beginning at 671, the N terminus as determined by Eppler et al. (13). These results demonstrate that gp7 is indeed processed and that this processing does not occur in a mutant lacking OpdA.

The processing of the *opdA*-independent phage mutants was studied in a similar way (Fig. 5). None of the mutant proteins was cleaved in the *opdA* host. The gp7 protein from mutant 1a was processed in the infected *opdA*$^+$ host, but gp7 from mutants 3a and 4a was not. Protein gp7 from mutant 3a particles is smaller than the unprocessed wild-type gp7 and larger than the processed form, consistent with the nucleotide sequence, which predicts a protein 13 amino acids shorter than the wild-type protein. These results show that all three mutations lead to proteins that function more effectively without proteolytic cleavage than the uncleaved wild-type protein. They do not alter the gene product in such a way that it can be cleaved by another protease.

Expression of gp7. To study the function and processing of gp7 in the absence of other phage proteins, plasmids which allowed the expression of the parental (P22 *c2*H5) (pCM187) and mutant 4a (pCM172) gene *7* under control of the *trc* promoter were constructed. Plasmid pCM187 contains a 1-kb fragment of phage DNA which encodes all of wild-type gp7 and a 9,000-MW amino-terminal fragment of gp20. Plasmid pCM172 contains a 2.8-kb fragment of phage DNA which encodes the carboxy-terminal half of gp26 (mw, ~15,000), all of gp14 (MW, 17,000), all of the mutant gp7 (MW 23,400), and a 26,000-MW amino-terminal fragment of gp20. The marker rescue experiments had shown that the mutants were able to confer their *opdA*-independent plaque phenotype when they were recombined into the phage genome. To determine whether the mutant gene *7* could confer its *opdA*-independent phenotype when expressed from a plasmid and not from the phage itself, pCM172 was introduced into TN3101 (*opdA10*::Mu*dJ*). P22 *c2*H5 made normal large plaques on a lawn of TN3101/pCM172 when expression of the mutant gene *7* was induced by the presence in the medium of IPTG, the inducer of the *trc* promoter, but made small plaques when expression was not induced. We conclude that the mutant gene *7* product is expressed from

```
   1 AAT AAA GGC GTA TTC GAT GCT GAC CTG ACA TTC GCC GTT AGC GAT ACT TAC ACG CAA TCT GAA ATC CAG GCT ATA GCC
     Asn Lys Gly Val Phe Asp Ala Asp Leu Thr Phe Ala Val Ser Asp Thr Tyr Thr Gln Ser Glu Ile Gln Ala Ile Ala

  79 AAT GCT CTA ATT ACT GAG CGT CGG CGC ACT AAG GCT TTG GAA GAC GCC TTG CGT GCA CAT GGG TTG ATT GAT TA ATG ATT
     Asn Ala Leu Ile Thr Glu Arg Arg Arg Thr Lys Ala Leu Glu Asp Ala Leu Arg Ala His Gly Leu Ile Asp    Met Ile  2

 159 ACA TTC ACT CCA ACA CGC AAC ATC GAC CTG ATA GAA ATG GTT GGC AAC CAC CCC GAC ATC ATT GCC GGA AGC AAC AAC
     Thr Phe Thr Pro Thr Arg Asn Ile Asp Leu Ile Glu Met Val Gly Asn His Pro Asp Ile Ile Ala Gly Ser Asn Asn   28

 237 GGT GAC GGA TAC GAC TAC AAG CCT GAG TGT CGT TAC TTT GAA GTG AAC GTA CAT GGT CAG TTC GGT GGC ATC GTG TAT
     Gly Asp Gly Tyr Asp Tyr Lys Pro Glu Cys Arg Tyr Phe Glu Val Asn Val His Gly Gln Phe Gly Gly Ile Val Tyr   54

 315 TAC AAC GAG ATT CAG CCG ATG ACC TTT GAC TGC CAC GCC ATG TAC CTG CCT GAG ATT CGC GGA TTC AGT AAG GAA ATC
     Tyr Asn Glu Ile Gln Pro Met Thr Phe Asp Cys His Ala Met Tyr Leu Pro Glu Ile Arg Gly Phe Ser Lys Glu Ile   80

                                        (14am H600,H611) T
 393 GGA CTG GCG TTC TGG CGA TAT ATT CTC ACC AAT ACC ACC GTT CAG TGC GTT ACA TCA TTT GCT GCA CGC AAA TTT GCG
     Gly Leu Ala Phe Trp Arg Tyr Ile Leu Thr Asn Thr Thr Val Gln Cys Val Thr Ser Phe Ala Ala Arg Lys Phe Ala  106

 471 GAC GGT CAG ATG TAC TGC GCA ATG ATT GGC CTT AAG CGT GTG GGA ACC ATC AAG AAA TAC TTC AAA GGC GTA GAT GAC
     Asp Gly Gln Met Tyr Cys Ala Met Ile Gly Leu Lys Arg Val Gly Thr Ile Lys Lys Tyr Phe Lys Gly Val Asp Asp  132

                          AvaI
 549 GTG ACG TTT TAC GCC GCC ACC CGA GAA GAG TTA ACC GAA TTA CTG AAT AAC GGG AGA TAAAC ATG TTA CAT GCA TTT ACG
     Val Thr Phe Tyr Ala Ala Thr Arg Glu Glu Leu Thr Glu Leu Leu Asn Asn Gly Arg       Met Leu His Ala Phe Thr   6

 629 CTG GGC AGG AAA CTG CGC GGT GAG GAA CCT TCT TAT CCT GAA AAA GGC GGT AAA GGT GGC GCA GAT AAA AGC GCA AAG
     Leu Gly Arg Lys Leu Arg Gly Glu Glu Pro Ser Tyr Pro Glu Lys Gly Gly Lys Gly Gly Ala Asp Lys Ser Ala Lys   32

 707 TAT GCA GCA GAA GCG CAA AAG TAT GCC GCA GAC CTG CAA AAC CAG CAG TTC AAT ACC ATC ATG AAC AAC CTG AAG CCG
     Tyr Ala Ala Glu Ala Gln Lys Tyr Ala Ala Asp Leu Gln Asn Gln Gln Phe Asn Thr Ile Met Asn Asn Leu Lys Pro   58

 785 TTT ACT CCT CTG GCA GAT AAG TAT ATC GGC AGT CTT GAA GGT TTA TCG TCT CTC GAA GGT CAG GGG CAG GCG CTT AAT
     Phe Thr Pro Leu Ala Asp Lys Tyr Ile Gly Ser Leu Glu Gly Leu Ser Ser Leu Glu Gly Gln Gly Gln Ala Leu Asn   84

 863 AAT TAC TAT AAC TCC CAA CAA TAC CAG GAC CTT GCG GGG CAG GCT CGC TAT CAG AAT CTG GCA GCG GCA GAA GCA ACA
     Asn Tyr Tyr Asn Ser Gln Gln Tyr Gln Asp Leu Ala Gly Gln Ala Arg Tyr Gln Asn Leu Ala Ala Ala Glu Ala Thr  110

 941 GGT GGC CTG GGT TCT ACA GCG ACC AGT AAC CAG CTT TCA GCA ATC GCC CCA ACA CTT GGT CAG CAA TGG CTG TCA GGT
     Gly Gly Leu Gly Ser Thr Ala Thr Ser Asn Gln Leu Ser Ala Ile Ala Pro Thr Leu Gly Gln Gln Trp Leu Ser Gly  136

     T (7am H1035)                                                  (7am H1375) T
1019 CAG ATG AAT AAC TAT CAG AAC CTT GCA AAT ATT GGT CTT GGT GCG CTT CAG GGG CAG GCA AAC GCC GGA CAG ACA TAT
     Gln Met Asn Asn Tyr Gln Asn Leu Ala Asn Ile Gly Leu Gly Ala Leu Gln Gly Gln Ala Asn Ala Gly Gln Thr Tyr  162

1097 GCC AAC AAT ATG AGT CAG ATT TCA CAG CAA AGC GCG GCT CTT GCA GCG GCA AAT GCC AAC AGA CCA TCA GCT ATG CAA
     Ala Asn Asn Met Ser Gln Ile Ser Gln Gln Ser Ala Ala Leu Ala Ala Ala Asn Ala Asn Arg Pro Ser Ala Met Gln  188

1175 TCT GCT ATT GGC GGA GGT GCG TCT GGT GCG ATT GCT GGG GCT GGA CTT GCG AAA TTA ATT GGT TCA TCA ACT CCG TGG
     Ser Ala Ile Gly Gly Gly Ala Ser Gly Ala Ile Ala Gly Ala Gly Leu Ala Lys Leu Ile Gly Ser Ser Thr Pro Trp  214

1253 GGT GCT GCC ATC GGT GGT GGT ATT GGT CTG CTT GGC TCG TTG TTT TAAGGGGTAATCA ATG GCT ACG TGG CAG CAG GGC
     Gly Ala Ala Ile Gly Gly Gly Ile Gly Leu Leu Gly Ser Leu Phe              Met Ala Thr Trp Gln Gln Gly

1332 ATT AAT TCA GGT GGT TTT CTG GCT GGC ATT GGT GCG CAA AAT GAG AAC GCA CCA AAG GCA AGA GAT ATT AAC GCA ACG
     Ile Asn Ser Gly Gly Phe Leu Ala Gly Ile Gly Ala Gln Asn Glu Asn Ala Pro Lys Ala Arg Asp Ile Asn Ala Thr

1410 CTG GGT CTG ATT CGC GAA AAC AAT GAT TTA GCC CGT TCA GGC GCT AAT AAT GTG GCT TTA ACA GGG CTG CGT GGT CTG
     Leu Gly Leu Ile Arg Glu Asn Asn Asp Leu Ala Arg Ser Gly Ala Asn Asn Val Ala Leu Thr Gly Leu Arg Gly Leu

1488 GCT GGC GTT GCT GAT ATT TAT AAC CAG GAA CAG CAA CAG AAA GCG CTA AAC GCA TTC AAC CAG GTT CAT GCC AAC GCA
     Ala Gly Val Ala Asp Ile Tyr Asn Gln Glu Gln Gln Gln Lys Ala Leu Asn Ala Phe Asn Gln Val His Ala Asn Ala

                 BstEII
1566 TGG GCT ACT GGT GAC CCG TCT
     Trp Ala Thr Gly Asp Pro Ser
```

FIG. 3. Nucleotide sequence and predicted ORFs. The gene 14 ORF extends from nucleotide 153 to 605. The gene 7 ORF extends from nucleotide 611 to 1297. The gene 20 ORF begins at nucleotide 1311. Amber mutations sequenced are indicated above the nucleotide sequence. The underlined amino acid sequence indicates the region of gp7 removed by processing.

the plasmid and the mutant allele is dominant to the wild-type allele. To further confirm that expression of the mutant gp7 was responsible for the *opdA*-independent phenotype, transposon Tn*1000* insertions in pCM172 were isolated. Five insertions which eliminated the ability of the plasmid to confer the *opdA*-independent phenotype were isolated and localized by restriction mapping. One was in the vector, between the promoter and the insert. The other four were located within the *Ava*I-*Bst*EII fragment which contains gene 7.

Figure 6 shows the expression of wild-type gp7 from plasmid pCM187 in *opdA* and *opdA*$^+$ cells. Only the processed form is seen in the *opdA*$^+$ strain, suggesting that the initial processing is rapid (half-life, <5 min) and that it does not require any other phage proteins. Only the unprocessed form is seen in the *opdA* host. The vector-only control expressed neither form (data not shown). The gp7 protein is rapidly degraded in both *opdA* and *opdA*$^+$ cells. The half-lives of mature gp7 in *opdA*$^+$ and *opdA* cells were measured by quantitating the radioactivity in the gp7 band with a Molecular Dynamics PhosphorImager and normalizing it to an unchanging band in each lane. The half-lives of gp7 in the *opdA*$^+$ and *opdA* hosts were 2.5 and 3 min, respectively.

```
(3a)                                                                  ──────────
(4a)                                                                      A
(1a)                                    A                             G
(WT) CGGGAGATAAAC ATG TTA CAT GCA TTT ACG CTG GGC AGG AAA CTG CGC GGT GAG GAA CCT
(WT)              Met Leu His Ala Phe Thr Leu Gly Arg Lys Leu Arg Gly Glu Glu Pro
(1a)                                    Lys                           Gly
(4a)                                                                      Lys
(3a)                                                                  ──────────

(3a) ──────────────────────────────────────────
(4a)              A
(1a)
(WT) TCT TAT CCT GAA AAA GGC GGT AAA GGT GGC GCA GAT AAA AGC GCA
(WT) Ser Tyr Pro Glu Lys Gly Gly Lys Gly Gly Ala Asp Lys Ser Ala
(1a)
(4a)              Lys
(3a) ──────────────────────────────────────────
```

FIG. 4. Amino-terminal region of gene 7 from mutant phages. The nucleotide sequence (nucleotide 599 to 703) and predicted amino acid sequence of wild-type P22 are presented. The nucleotide changes in the mutant phage and the corresponding amino acid changes are indicated. The solid line indicate the extent of the deletion in P22 3a. The amino terminus of mature gp7, as determined by Eppler et al. (13), is shown in italics.

To study the processing step directly, the wild-type gene 7 was cloned behind the phage T7 promoter (pCM193) and expressed in *S. typhimurium* TN3685. TN3685 contains the phage T7 RNA polymerase gene under inducible control in its chromosome. After the expression of the T7 polymerase was induced and the cells were treated with rifampin to inhibit the *S. typhimurium* RNA polymerase, gp7 was labelled with a 1-min pulse of ^{14}C-amino acids and then chased with an excess of Casamino Acids. The autoradiogram in Fig. 7 shows that the processing is very rapid.

The proteolytic instability of gp7 was unexpected and intriguing. Preliminary studies of gp7 degradation were performed to determine (i) whether gp7 degradation was dependent on *lon* protease and (ii) whether the mutation in mutant 4a stabilized gp7. The stability of many abnormal and foreign proteins is increased in *lon* strains. To determine whether the degradation of gp7 was dependent on *lon* protease,

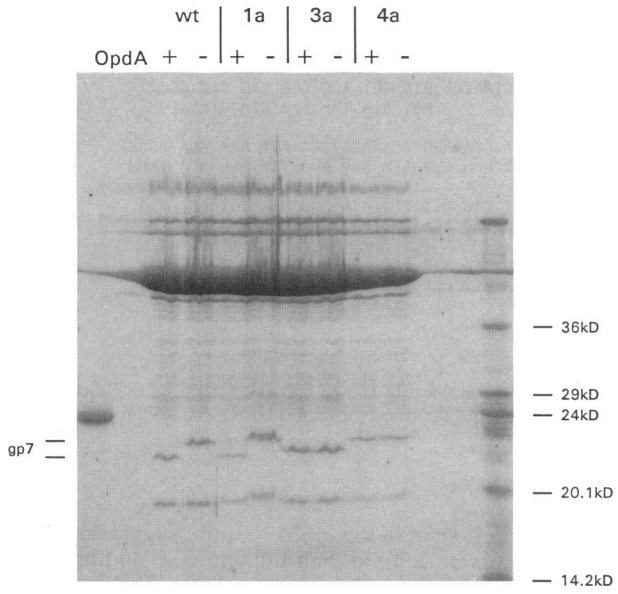

FIG. 5. SDS-PAGE of proteins from P22 particles grown on *opdA*$^+$ and *opdA* hosts. Phage was grown on TN1379 (*opdA*$^+$) or TN3101 (*opdA*) and purified by $CsCl_2$ gradient centrifugation. Proteins were separated on an SDS–12% polyacrylamide slab gel by the buffer system of Laemmli (24) and visualized by staining the gel with Coomassie blue R250. wt (wild type) is P22 *c2*H5 *13am*H101, 1a is P22 7-1a, 3a is P22 7-3a, 4a is P22 7-4a. +, phage was prepared from TN1379 (*opdA*$^+$); −, phage was prepared from TN3101 (*opdA10*::MudJ).

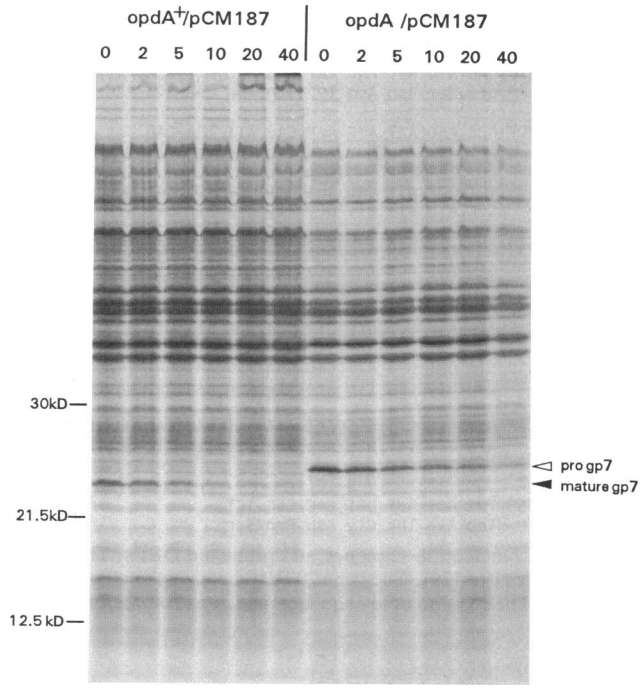

FIG. 6. Expression and degradation of wild-type gp7 from pCM187 in *opdA*$^+$ (TN1379) and *opdA* (TN3101) hosts. Protein gp7 expression was induced by adding IPTG. Proteins were labelled with a 5-min pulse of ^{14}C-amino acids and chased with unlabelled Casamino Acids. Samples were removed and placed on ice 0, 2, 5, 10, 20, and 40 min after the pulse, and cells were pelleted, suspended in SDS sample buffer, and heated to 95°C for 5 min. Proteins were separated on an SDS–12% polyacrylamide slab gel by the Laemmli (24) buffer system and detected by autoradiography. P22 gp7 was identified by its comigration with gp7 from phage particles, by its absence in the vector-only controls (data not shown), and by its altered mobility when expressed in *opdA* and *opdA*$^+$ hosts.

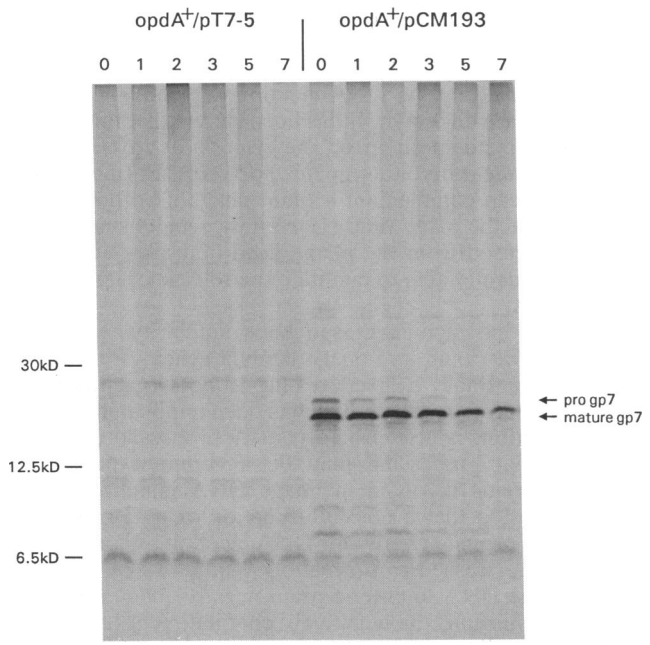

FIG. 7. Processing of gp7. TN3685 contains an IPTG-inducible chromosomal T7 RNA polymerase gene and pCM193, a plasmid carrying the wild-type P22 gene 7 behind a T7 RNA polymerase-specific promoter. The T7 RNA polymerase was induced with IPTG, rifampin was added, and then ^{14}C-amino acids were added. After 1 min, an excess of unlabelled Casamino Acids was added. Samples were removed and placed on ice 0, 1, 2, 3, 5, and 7 min after the pulse. Cells were pelleted, resuspended in SDS sample buffer, and heated to 95°C for 5 min. Proteins were separated on SDS–10% polyacrylamide–Tris–tricine gels (34) and detected by autoradiography.

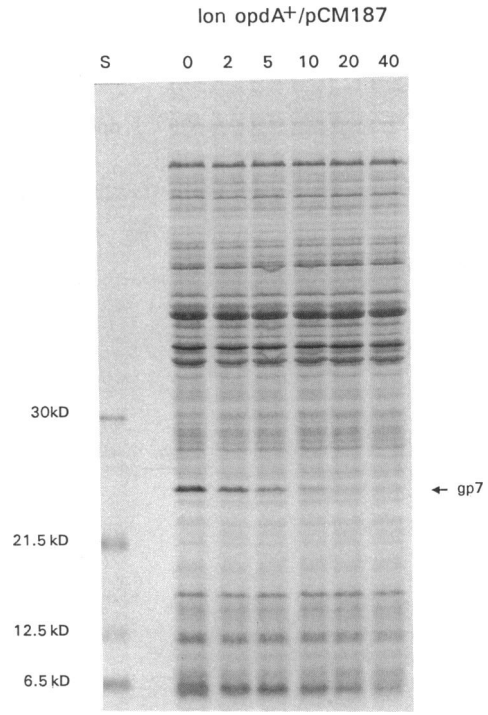

FIG. 8. Degradation of gp7 is *lon* independent. See the legend to Fig. 6 for a description of the experiment. The host strain for pCM187 was TN2684 (*lon-101*).

pCM187 was moved into TN2684 (*S. typhimurium lon-101*). Protein gp7 was pulse-labelled with ^{14}C-amino acids and chased with Casamino Acids. Figure 8 shows that gp7 was rapidly degraded in the *lon* strain, demonstrating that gp7 degradation was not *lon* dependent. To study the effect of the gene 7 mutations in mutant 4a on the stability of gp7, the mutant 4a gp7 protein was expressed from the *trc* promoter in pCM185. Plasmid pCM185 contains a 1.5-kb fragment of mutant 4a DNA which encodes the 23,400-MW mutant gp7 and a 26,000-MW amino-terminal fragment of gp20. Although mutant 4a gp7 is not processed, it appeared to be degraded as rapidly as the processed wild-type gp7 in both *opdA*$^+$ and *opdA* backgrounds (Fig. 9).

DISCUSSION

The major conclusions of this study are the following. (i) OpdA is required for the normal growth of phage P22. (ii) Phage P22 gp7 undergoes a previously unrecognized post-translational processing in which 20 amino acids are removed from the N terminus of the protein. (iii) This processing requires the host endopeptidase OpdA and occurs in the absence of other phage proteins. (iv) Mutations which increase the efficiency of unprocessed gp7 function are located in the N-terminal portion of gp7. (v) Phage protein gp7 is rapidly degraded when expressed in the absence of phage infection. This degradation occurs whether or not gp7 is processed and is independent of the *lon* protease.

The only host mutations previously reported to affect the growth of phage P22 are those reported by Joshi et al. (23). These mutations were selected as conferring thiolutin resistance. Strains carrying these mutations do not support the normal growth of P22 at an elevated temperature (40°C). Although phages grown on one class of these mutants appeared morphologically normal by electron microscopy, they failed to inject their DNA into the host. We have not tested the thiolutin sensitivity of our *opdA* strains, and the thiolutin resistance mutations in *S. typhimurium* have not been mapped. Other thiolutin resistance mutations have been mapped to 11 and 85 map units in *E. coli* (36). *opdA* maps at 76 map units on the *S. typhimurium* chromosome (9), and *prlC*, the *E. coli* homolog of *opdA* (10), maps at 71 map units on the *E. coli* chromosome (39). Although the relationship between the thiolutin resistance mutations and *opdA* is unknown, the map positions of the *E. coli* mutations suggest that they affect different genes.

Although proteolytic processing is a common aspect of phage development, previous studies have reported no proteolytic processing of P22 proteins during morphogenesis (2, 6). Whereas some of the phage lambda minor coat protein, gpC, is cleaved and joined to the major coat protein gpE (17, 18), the P22 major coat protein gp5 undergoes no proteolytic modifications (6). Unlike the lambda scaffolding protein gpNu3, which is degraded (30), the P22 scaffolding protein gp8 is not degraded but is recycled (6). Not only did earlier studies of proteolytic processing in P22 (2, 6) fail to detect processing of gp7, but because of its size and low abundance, they did not even detect mature gp7.

The proteolytic processing of gp7 is both independent of other phage proteins and dependent on the host protein OpdA. The simplest explanation for this is that the endopeptidase OpdA is directly responsible for the proteolytic pro-

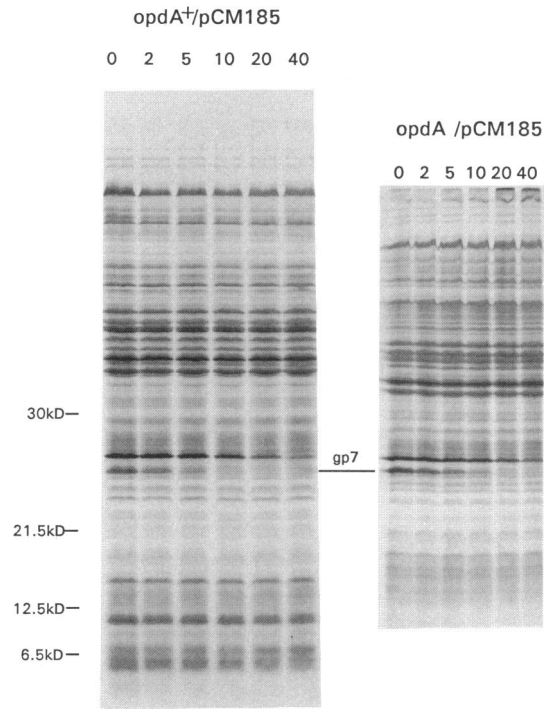

FIG. 9. Mutant gp7 from P22 7-4a, although not processed, is still rapidly degraded in an *opdA*-independent manner. See the legend to Fig. 6 for a description of the experiment. The plasmid was pCM185, which carries gene *7* from P22 7-4a and a fragment of gene *20* from pSE380. The host strains were TN1379 (*opdA*$^+$) and TN3101 (*opdA*). The 26,000-MW protein above gp7 is presumably an amino-terminal fragment of gp20 encoded by the insert DNA.

cessing of gp7 by cleaving the bond between Glu-20 and Lys-21, two charged residues. However, limited in vitro specificity studies, mainly with the lipoprotein signal peptide as a substrate, have indicated that OpdA prefers an Ala or Gly on either side of the scissile bond (27, 41). It should be noted that there is no Glu-Lys site or even two adjacent charged residues in the lipoprotein signal peptide, so studies with this substrate provide no information about the specificity of OpdA for such a site. Even if OpdA fails to attack the Glu-Lys bond in vitro, it is possible that this does not accurately reflect its substrate specificity in vivo.

It has also been reported that OpdA from *E. coli* does not attack the lipoprotein signal peptide while it is attached to the precursor protein (27). This suggests that OpdA prefers small polypeptide substrates and that it would be unlikely to attack the unprocessed gp7. Once again, however, these results are from in vitro studies, and the in vivo situation may be different. It is also possible that OpdA is not directly responsible for the removal of the N-terminal peptide. It may be indirectly required for gp7 processing, required for activation of another protease, or required for maintaining the unprocessed gp7 in a conformation susceptible to processing.

The phage proteins gp7, gp16, and gp20 are required for ejection of the phage DNA from the phage particle and injection of that DNA into the host (2, 19, 20, 29). Although these proteins are incorporated into proheads early in development before the DNA is packaged, phage particles lacking any one of these proteins are morphologically indistinguishable from wild-type particles (2, 29). Inactivation studies using DNA-intercalating agents have shown that in the mature phage particle, gp7, gp16, and gp20 are in close association with the phage DNA (4). They are all lost from the phage head during DNA ejection (20), but their subsequent fates are unknown. It has been shown that the ejection of gp7 is dependent on gp16. The ejection of gp16 however, is not dependent on either gp7 or gp20 (20). Furthermore, a *16*$^+$ phage can complement a *16am* phage if both infect the same cell at the same time (19). These results suggest that gp16 is released from the phage head first after adsorption and subsequently acts to facilitate the release of gp7 and the phage DNA.

Although no significant sequence similarity was found between gene *7* and any phage lambda genes, or any other genes in GenBank, it is interesting to note that the phage lambda tail protein gpH, which has been implicated in phage DNA injection, is also proteolytically processed (17). This suggests that processing may be a common occurrence among proteins involved in phage DNA injection. It has been suggested that a possible function of gpH processing might be to provide energy for DNA ejection by forcing gpH to assume a stressed conformation (17).

For phage DNA to enter a gram-negative bacterium such as *S. typhimurium*, the negatively charged, very large (28.6-MDa) DNA molecule must traverse two phospholipid bilayers and the periplasmic space. How this is done is unknown. Phage P22 adsorbs to the lipopolysaccharide (LPS) by its tail spike. The tail spike protein is an active endorhamnosidase and digests the polysaccharide side chains of the LPS (21). Presumably, this allows access to an unidentified secondary receptor in the outer membrane. It is possible that by interacting with this outer membrane receptor, the phage gains access to the periplasm. Subsequently, the DNA must be released and must pass through the periplasm and the inner membrane. Therefore, possible roles for injection proteins include releasing the DNA from the phage particle, directing it across the periplasm, protecting it from degradation, and getting it through the inner membrane. It is not known whether P22 pirates a cellular transport system to gain entry to the cytoplasm or whether the injection proteins themselves are sufficient to allow the phage DNA to enter the cytoplasm.

Several elements of the gene *7* sequence might provide clues to the function of this protein. The mature N terminus has a large number of basic residues. Five of the 19 N-terminal amino acids are lysines, resulting in a net charge of +3 (predicted pI of 9.5) for this N-terminal peptide. When projected onto a helical wheel (35), the basic residues concentrate on the same face of the helix. The *opdA*-independent mutations all make the unprocessed N terminus more basic than the wild-type unprocessed N terminus. The N-terminal peptide (20 amino acids) of the wild-type unprocessed protein has a predicted pI of 7.01, whereas the predicted pIs of the first 20 amino acids in the unprocessed N termini of mutant phages 1a, 3a, and 4a are 9.61, 10.36, and 10.36, respectively. This suggests that a basic N terminus may be important to gp7 function.

The C-terminal half of gp7, on the other hand, is rather hydrophobic. There are two especially hydrophobic regions at the far C terminus, residues 190 to 205 and 214 to 229, which are separated by the positively charged Lys-206 and are predicted by the method of Eisenberg et al. (12) to be potential membrane-spanning regions.

These findings suggest two possible functions for gp7. (i) The basic N terminus of gp7 might bind to the phage DNA, and the hydrophobic C terminus might be inserted into the

inner membrane of the host cell, perhaps directing the DNA to its entry site. (ii) The function of the positively charged N terminus may be not to bind DNA but to direct the protein to the negatively charged phospholipid head groups of the membrane. Subsequently, the hydrophobic C terminus would insert itself into the membrane. This would be similar to the insertion of bacteriophage M13 procoat protein into the cytoplasmic membrane (14).

The N-terminal peptide of the unprocessed protein contains 6 charged residues in 20 amino acids. However there is no net charge (predicted pI of 7.01). This neutral, hydrophilic N-terminal segment might keep the relatively hydrophobic gp7 protein soluble and in an assembly-competent conformation. It might also prevent the premature association of gp7 with DNA or block its insertion into the membrane. Removal of the N-terminal peptide in an *opdA*-dependent manner might allow the protein to fold into its active conformation and expose the mature basic N terminus. Obviously, further experiments are necessary to elucidate the function and mechanism of action of gp7.

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