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Essential Role of a Sodium Dodecyl Sulfate-Resistant Protein IV Multimer in Assembly-Export of Filamentous Phage

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Filamentous phage f1 encodes protein IV (pIV), a protein essential for phage morphogenesis that localizes to the outer membrane of *Escherichia coli*, where it is found as a multimer of 10 to 12 subunits. Introduction of internal His or Strep affinity tags at different sites in pIV interfered with its function to a variable extent. A spontaneous second-site suppressor mutation in gene *IV* allowed several different insertion mutants to function. The identical mutation was also isolated as a suppressor of a multimerization-defective missense mutation. A high-molecular-mass pIV species is the predominant form of pIV present in cells. This species is stable in 4% sodium dodecyl sulfate at temperatures up to 65°C and is largely preserved at 100°C in Laemmli protein sample buffer containing 4% sodium dodecyl sulfate. The suppressor mutation makes the high-molecular-mass form of wild-type pIV extremely resistant to dissociation, and it stabilizes the high-molecular-mass form of several mutant pIV proteins to extents that correlate with their level of function. Mixed multimers of pIV^{f1} and pIV^{Ike} also remain associated during heating in sodium dodecyl sulfate-containing buffers. Thus, sodium dodecyl sulfate- and heat-resistant high-molecular-mass pIV is derived from pIV multimer and reflects the physiologically relevant form of the protein essential for assembly-export.

The means by which large macromolecular complexes move across biological membranes is poorly understood. Assembly-export of filamentous bacteriophages is a system in which this problem can be explored in detail by combined genetic and biochemical approaches. Phage f1 assembly and export are concerted processes that occur at the membrane of infected *Escherichia coli* (reviewed in references 27 and 40). In addition to essential capsid proteins (protein III [pIII], pVI, pVII, pVIII, and pIX) and the phage DNA-pV complex (8, 27), assembly requires host-encoded thioredoxin (43) and three phage-encoded proteins not found in completed virus particles. These morphogenetic factors are inner membrane proteins pI and pXI (formerly pI*) (33) and pIV, an integral protein of the outer membrane (2). Outer membrane protein pIV forms a multimer of 10 to 12 identical subunits (21). The C-terminal domain of pIV (≈200 amino acids) has a high content of polar amino acids and is predicted to have extensive β-sheet (2), which is typical for outer membrane proteins such as porins (reviewed in reference 31). This region of pIV is homologous to regions of outer membrane proteins found in a variety of gram-negative bacteria (13, 19, 38, 42). One feature common to all of these proteins is their involvement in the transfer of enzymes, toxins, pili, and other macromolecules across outer membranes (39). We have proposed that the pIV multimer forms a channel through which the assembling phage passes as it is extruded to the external milieu (2, 21). The bacterial proteins homologous to pIV may also form export channels.

At least one, and typically several, inner membrane proteins, including one with ATP-binding activity, participate in these bacterial export systems. In the phage system, genetic analysis has revealed a role for the ≈250-amino-acid N-terminal cytoplasmic domain of pI in recognition of the f1 packaging site DNA, thioredoxin, and ATP (10, 43, 44). Thus, pI likely participates in phage assembly-export from the initiation step. Gene *XI* is overlapping and in frame with the final one-third of gene *I* (33). It encodes the membrane anchor and C-terminal periplasmic domain of pI but not the cytoplasmic part (16). Mutational evidence suggests that important interactions occur between the C-terminal domain of pI (and/or pXI) and the N terminus of pIV in the periplasm (36, 37). We have proposed that the C-terminal periplasmic domain of pI may regulate the gating of the hypothetical pIV channel (39, 40).

As a prelude to purifying native pIV multimeric complexes for biochemical and physical studies, we have isolated f1 phages with His or Strep affinity tag inserts in gene *IV*. During the course of characterizing these pIV insertion mutants by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot (immunoblot) analysis, we discovered an unusual property of the wild-type protein: it migrates as two species, one the monomeric form (45 kDa; mono-pIV) and the other a slowly migrating high-molecular-mass (HMM) form that remains trapped in the stacking gel (HMM-pIV). Like pIV, its homolog in *Neisseria gonorrhoeae*, OMP-MC (or PilQ), forms a large homomultimer. Depending on conditions of sample preparation, OMP-MC/PilQ migrates either at one or two positions during SDS-PAGE (7, 30). A description of the pIV homolog YscC of *Yersinia pestis* (32) suggests that this protein also migrates as two forms. Since a dual-migration pattern had not been observed for pIV before, we have investigated this phenomenon in greater detail.

Here we report the unusual electrophoretic behavior of pIV and its significance with regard to pIV structure and function, and we describe a novel mutation that confers extreme SDS and heat stability on multimeric pIV (HMM-pIV).

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and culture conditions. Bacterial strains and bacteriophages used are listed in Table 1. The system of amino acid numbering used throughout is for the mature wild-type pIV protein (minus the 21 signal sequence residues). pIV His tag mutants are named according to the number of histidine residues added and the codon after which they were added (e.g., A_{308}His2 has two histidines added after alanine 308; see Table 1 for further details). Strain K1472 was constructed by P1-mediated transduction (26) of

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TABLE 1. *E. coli* strains and bacteriophages used

Strain or phage	Protein IV	Relevant characteristic(s)[a]	Reference, source, or construction[b]
E. coli K-12 strains			
A528		F^+ (λ *c*I857)	36
A627		A528 (pPMR84, λ P_L gene IV^{Ike}, Ap^r)	36
K38		HfrC sup^0	Laboratory collection
K1053		F^+ *dut ung*	38
K1472		Δ(*pspABC*::Kan^r)	This work
K1478		K38 *recA56* $lacI^q$ (pNL200, P_{tac} gene *IV*, $lacI^q$, Cm^r)	This work
K1479		K38 *recA56* $lacI^q$ (pGZ119EH, P_{tac}, $lacI^q$, Cm^r cloning vector)	Laboratory collection
f1 phages			
R352	Wild type	*Sal*I site after gene *IV*	Laboratory collection
R482	Unprocessed	Nonexported (4-codon deletion in the pIV signal sequence)	2
R484		1,053-bp deletion in gene *IV*	2
R534	Wild type	Produces pIV at 1/10 the f1 rate (altered ribosome binding site)	38
R616	P375A	Unstable, multimerization defective	38
R642	Wild type	R352 with *Eco*RI site before gene *IV*	SDM (NALI-7)
R643	Wild type	R642 with *Xba*I site in gene *IV*	SDM (NALI-3)
R649	A_{308}His2	R643 with SAH2 inserted after A-308	SDM (NALI-8)
R652	P375A S318I	R616 with S318I (Stm)	SM
R661	A_{308}His5 S318I	R649 with H5 in place of H2, and S318I (Stm)	IM (NALI-14/-15) and SM
R662	A_{308}His7 S318I	R661 with H7 in place of H5	IM (NALI-26/-27)
R663	D_{347}His2	R643 with SAH2 after D-347	SDM (NALI-17)
R664	D_{347}His2 S318I	R663 with S318I (Stm)	SM
R666	D_{347}His7 S318I	R664 with H7 in place of H2	IM (NALI-26/-27)
R670	A_{308}Strep-tag S318I	R662 with Strep tag (SAWRHPQFGGH) in place of SAH7	IM (NALI-19/-20)
R671	D_{347}Strep-tag S318I	R666 with Strep tag (SAWRHPQFGGH) in place of SAH7	IM (NALI-19/-20)
R672	S318I	R643 with S318I (Stm)	SDM (NALI-22)
R673	S_{258}His2	R643 with SAH2 after S-258	SDM (NALI-13)
R674	N_{269}His2	R643 with SAH2G after N-269 and loss of V-270	SDM (NALI-16)
R675	Q_{336}His2	R643 with SAH2G after Q-336	SDM (NALI-18)

[a] Amino acids added or deleted in pIV are indicated by the one-letter code.
[b] Abbreviations: IM, insertion mutagenesis; SDM, site-directed mutagenesis; SM, spontaneous mutation. The names of oligonucleotides used to construct phages are given in parentheses (see also Fig. 1; for sequences, refer to Table 2).

MC1061 (4), using a P1 lysate grown on the Δ(*pspABC*::Kan^r) strain J134 (53); recipients were selected on LB agar containing 25 μg of kanamycin per ml.

Host bacteria for f1 (strain K38, unless stated otherwise) were grown in fortified broth (54) with aeration at 37°C to 4×10^8 cells per ml, infected with the indicated phages (multiplicity of infection of 20 to 40), grown usually for 30 min, chilled on ice, collected by centrifugation (5,000 × *g*, 4°C, 8 min), washed once in cold Tris-saline (50 mM Tris HCl [pH 8.0], 100 mM NaCl), and repelleted. For isotopic labeling of proteins with [^{35}S]methionine, cells were grown in minimal medium (52) supplemented with glucose, amino acids (except methionine), thiamine, and biotin as described previously (2). K38 (grown at 37°C) and A627 (grown at 33°C) were infected with phage for 25 min or mock infected. A627 was then shifted to 38°C for 5 min to induce expression of gene IV^{Ike}. Cells were labeled for 1 or 10 min with [^{35}S]methionine (per 0.1 ml of cells, 20 μCi; 1,000 Ci/mmol; Amersham). Under these conditions, all of the label is incorporated in the first minute and times thereafter constitute a chase period even though unlabeled methionine is not supplied exogenously. To recover native proteins, cells were chilled on ice and washed once with Tris-saline. To obtain denatured proteins, cells were precipitated with an equal volume of cold 10% (wt/vol) trichloroacetic acid and rinsed with acetone.

Analysis of proteins by immunoprecipitation, SDS-PAGE, and immunoblotting. Immunoprecipitations and coimmunoprecipitations of [^{35}S]methionine-labeled proteins were carried out as described before (21, 42). The anti-pIV^{f1}, anti-pIV^{Ike}, and anti-PspA sera were preincubated 1 h at room temperature with excess unlabeled SDS-denatured proteins of K38, A528, and K1472, respectively, to reduce the appearance of contaminating bands. For coimmunoprecipitation experiments requiring anti-pIV^{f1}, excess unlabeled proteins of A627 induced 1 h at 38°C and extracted in Triton X-100 were also added, and preincubation was overnight at 4°C; for anti-pIV^{Ike}, Triton X-100-extracted proteins of K1478 induced 1 h with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were used instead.

For analysis of unlabeled proteins, cells were lysed by heating at 100°C for 5 to 6 min in straight 4% SDS, in protein gel sample buffer (SB), or as stated in the text and briefly centrifuged. Standard SB contained 125 mM Tris HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol (B-ME), 0.01% bromophenol blue, and 20% glycerol. SB plus urea contains all components of SB except glycerol plus 4 to 8 M urea, as indicated in the text. To obtain native total membranes, cells were treated with lysozyme-EDTA and osmotic shock and centrifuged as described before (2). The membranes were solubilized either in 2% Triton X-100 (in a mixture of 5 mM EDTA, 50 mM Tris HCl [pH 8.0], 50 mM NaCl, and 100 Kallikrein units of aprotinin per ml) overnight at 4°C or in SDS or SB (for details of solubilization, see below). A Mini-Protean II electrophoresis unit (Bio-Rad, Hercules, Calif.) and SDS-containing 11% polyacrylamide gels (37.5:1 acrylamide/bisacrylamide [24]) were used throughout. Proteins from 10^7 to 10^8 cell equivalents were applied per lane following heating in SB at 100°C for 5 min, unless stated otherwise. Prestained molecular mass Rainbow markers for protein gels were purchased from Amersham (Arlington Heights, Ill.).

For immunoblot analysis, separated proteins were transferred either to membranes of nitrocellulose (NC; Protran; Schleicher & Schuell, Keene, N.H.), for qualitative comparisons, or to membranes of polyvinylidene fluoride (PVDF; Immobilon-P^{SQ}; Millipore Corp., Bedford, Mass.), for quantitative studies, at 100 V for 1 h, using a Mini Trans-blot apparatus (Bio-Rad). Cold transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol, 0.0125% SDS) and cooling were used. Unless otherwise indicated, detection was by enhanced chemiluminescence (Amersham). Methods for this procedure and alkaline phosphatase (Promega, Madison, Wis.) detection were those recommended by the manufacturers. Core streptavidin-alkaline phosphatase conjugate was purchased from Sigma (St. Louis, Mo.). Polyclonal anti-pIV serum from a rabbit (2) was used at a 1:10,000 dilution.

DNA and oligonucleotide methods. DNA restriction and modifying enzymes were purchased from New England Biolabs (Beverly, Mass.) or from Boehringer Mannheim (Indianapolis, Ind.). Plasmid pNL200 was constructed from *Bam*HI-linearized DNA of the P_{tac} cloning vector pGZ119EH (25) and a 1.4-kb *Bam*HI fragment carrying gene *IV* resected from pJLB4 (2), using standard molecular biological techniques (46).

For mutagenesis, single-stranded DNA was prepared by phenol extraction of phage grown on the *dut ung* strain K1053. DNA oligonucleotides listed in Table 2 (Operon Technologies, Inc., Alameda, Calif.) were phosphorylated with T4 polynucleotide kinase. The oligonucleotides (5 pmol) were annealed to 1 pmol of template in 20 μl in 40 mM Tris HCl (pH 7.5)–20 mM $MgCl_2$–50 mM NaCl, diluted 1:1 with extension buffer (consisting of 2.2 mM ATP, 100 μg of bovine serum albumin per ml, 0.5 mM dithiothreitol, and 0.5 mM all four deoxyribonucleoside triphosphates plus 2 U of T7 DNA polymerase and 400 U of T4 DNA ligase), and incubated 1 h at 37°C. For insertion mutagenesis, complementary 5′-phosphorylated oligonucleotides (0.1 pmol of each) were annealed as de-

TABLE 2. f1-specific oligonucleotides used for site-directed and insertion mutagenesis

Name	Sequence[a]	Position in f1[b]
NALI-3	5′-GAAAAATCTAGAGCATCAC-3′	4848
NALI-7	5′-CATTTGAATTCCCTTTTTTAATG-3′	4200
NALI-8	5′-TATTACCGCC**ATGGTGTGCACT**AGCCATTGCA-3′	5197
NALI-13	5′-CCCTTCTGACC**ATGGTGTGC**ACTAAGCGTAAG-3′	5045
NALI-14	5′-***TGCACATCATCACCAC***-3′	
NALI-15	5′-**CATGGTGGTGATGATG**-3′	
NALI-16	5′-CCAGTAATAAAAGGGCC**ATGGTGTGCA**CTATTCTGACCAAC-3′	5078
NALI-17	5′-GAGTCTGTCC**ATGGTGTGCACT**ATCACGCAAATTAAC-3′	5309
NALI-18	5′-GCAATACTTCT**GCCATGGTGTGCACT**TTGATTAGTAATAAC-3′	5276
NALI-19	5′-***TGCATGGCGCCCACCCGCAGTTCGGTGGC***-3′	
NALI-20	5′-**CATGGCCACCGAACTGCGGGTGGCGCCA**-3′	
NALI-22	5′-GCCTTGATGGTAATATCC-3′	5224
NALI-26	5′-***TGCACACCATCACCACCATCAC***-3′	
NALI-27	5′-**CATGGTGATGGTGGTGATGGTG**-3′	

[a] Complementary to the viral strand. Altered bases are underlined, and inserted bases are in boldface. Italics indicate sequences inserted into the viral strand.
[b] Nucleotide position in the f1 genome at the 3′ base of the oligonucleotide, numbered according to Hill and Petersen (18). Nucleotide positions are not given for insertion oligonucleotides since each was cloned at several positions.

scribed above and then ligated at 22°C for 2 to 4 h with *Apa*LI-*Nco*I-linearized, 5′-dephosphorylated double-stranded phage DNA (0.5 pmol) and T4 DNA ligase. Competent *dut*$^+$ *ung*$^+$ cells were transfected with portions (1 to 4 μl) of the DNA-oligonucleotide mixtures and plated in TB soft agar (1% tryptone, 0.1% yeast extract, 0.1% glucose, 0.8% NaCl, 0.7% agar) on TB plates containing 1.4% agar. Recipient cells were K38 or, in the case of gene *IV* mutants, a *recA* strain supplying pIV from plasmid pNL200 (K1478), and 250 μM IPTG was present during plating. Stocks were grown from single plaques, and phage DNAs were sequenced by using [α-^{35}S]dATP (New England Nuclear, Wilmington, Del.) and the 7-deaza-dGTP reagent kit with Sequenase enzyme according to protocols from United States Biochemical (Cleveland, Ohio). In some instances, candidate phage mutants were identified by plaque lift to NC (Schleicher & Schuell) and hybridization of the membranes with the appropriate 5′-γ-^{32}P-labeled mutagenic oligonucleotide as the probe.

Protein structure prediction. A prediction of pIV secondary structure was made by using the program Homology Derived Secondary Structure of Proteins, version 1.0 (35), made available by the European Molecular Biology Laboratory, Heidelberg, Germany. The pIV sequences of the following filamentous phages were used to construct the alignment: f1, fd, M13, Ike, and I2-2.

RESULTS

Isolation of insertion mutants of pIV. Several attempts to obtain quantities of purified pIV suitable for biochemical studies by conventional procedures have been unsuccessful. To facilitate its purification, we constructed f1 mutant phages with His (20) or Strep (48) affinity tags inserted into pIV. The proteins carrying an N- or C-terminal His tag functioned poorly in phage assembly (data not shown) and were not studied further. Earlier studies had demonstrated that C-terminal alterations destabilize pIV (2, 34). The N-terminal tag could have disturbed protein contacts inferred to occur between the N terminus of pIV and assembly protein pI (36, 37).

Internal tags were inserted after residues that the Rost and Sander (35) program predicted to reside in short loops separating β-strands (Fig. 1). A minimal His tag was inserted at five internal positions, using an oligonucleotide that also introduced two unique restriction sites (*Apa*LI and *Nco*I). The His2 insertion phage were isolated on a strain supplying pIV in *trans* from plasmid pNL200 (K1478), and the desired nucleotide changes were confirmed by DNA sequencing. One mutant, A_{308}His2, could grow in the absence of the pIV$^+$-supplying plasmid. It formed clear and slightly smaller plaques than wild-type phage, indicative of reduced pIV function. Two other mutants, N_{269}His2 and Q_{336}His2, appeared to be dominant interfering mutants. They formed small plaques with clear centers and produced low-titer liquid stocks, even though the pIV$^+$ plasmid was supplying wild-type levels of protein. No pure stocks of either could be obtained. Sequencing of these phage's DNA always revealed mixed populations, with most templates carrying new mutations in gene *IV* that eliminated the synthesis of the His tag proteins. By contrast, mutants S_{258}His2 and D_{347}His2 grew only with pIV$^+$ in *trans*, but they made normal-looking plaques, which indicated that the His tag pIVs did not interfere with the function of the wild-type protein. Consistent with this interpretation, these latter mutants were genetically stable.

To find improved variants of the four nonfunctional His2 mutants, high titers of phage were plated on a host not supplying pIV$^+$ (K38), and plaques were obtained at a frequency of 10^{-4} to 10^{-6}. One descendant of D_{347}His2 formed normal-size, slightly clear plaques, retained the His tag insert, and had

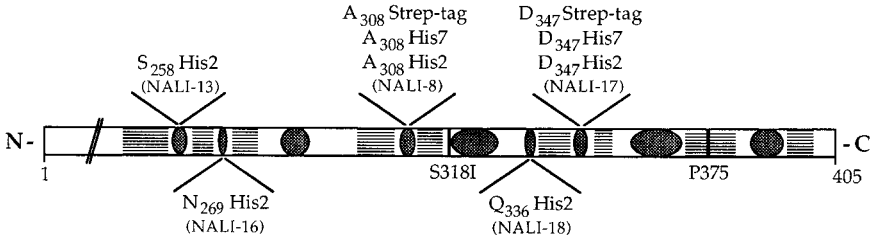

FIG. 1. Schematic representation of the C-terminal half of mature pIV, showing positions of affinity tag insertions. Segments predicted to form β-strands are distinguished by horizontal stripes, and cross-hatched ovals represent probable loops and β-turns. The positions of other mutations mentioned in the text are marked by vertical lines. Names of oligonucleotide primers used to insert bases specifying the His2 codons and restriction sites (*Apa*LI and *Nco*I) appear in parentheses. Complementary oligonucleotide pairs with *Apa*LI and *Nco*I ends were used to extend His2 inserts to His5 (NALI-14 and NALI-15), His7 (NALI-26 and NALI-27), or Strep tag (NALI-19 and NALI-20). The region after amino acid 240 is drawn approximately to scale.

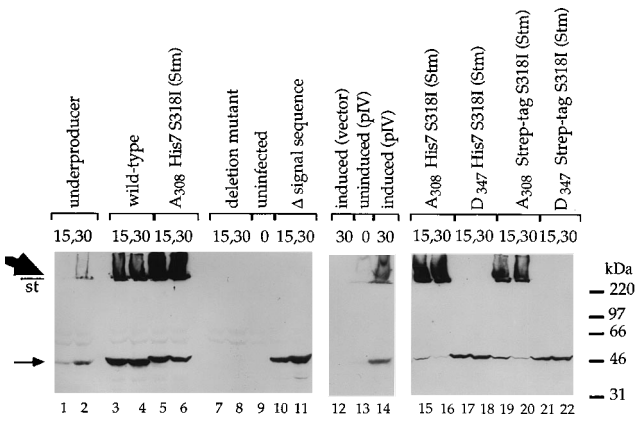

FIG. 2. Effects of gene *IV* mutations on the appearance of an HMM-pIV complex. pIV-producing cells were collected 0, 15, or 30 min (as indicated above the lanes) after infection by the indicated phages or induction with 1 mM IPTG and then heated in SB (100°C, 5 min). The proteins were separated by SDS-PAGE and transferred to NC. Blots were probed with anti-pIV serum. Small arrow, mono-pIV; large arrowhead, HMM-pIV complex; st, boundary of stacking gel. Lanes: 1 and 2, underproducer of wild-type pIV (R534); 3 and 4, wild-type pIV (R643); 5 and 6, pIV^{Stm} A_{308}His7 (R662); 7 and 8, pIV deletion mutant (R484); 9, uninfected K38; 10 and 11, pIV signal sequence mutant (R482); 12, induced K1479 carrying pGZ119EH (vector); 13, uninduced K1478 carrying pNL200 (P_{tac} gene *IV*); 14, induced K1478 carrying pNL200 (P_{tac} gene *IV*); 15 and 16, pIV^{Stm} A_{308}His7 (R662); 17 and 18, pIV^{Stm} D_{347}His7; 19 and 20, pIV^{Stm} A_{308}Strep-tag (R670); 21 and 22, pIV^{Stm} D_{347}Strep-tag (R671).

no gross changes in its DNA. Sequencing revealed a G-to-T nucleotide change at position 5235, in the vicinity of the insert, causing the amino acid replacement S318I in pIV. Mutants N_{269}His2 and Q_{336}His2 each yielded descendants that formed small irregular plaques; these were not studied further. Many other His2 progeny had recombined with the pIV supplier plasmid.

The His2 insert was extended to His5 or His7 or changed to encode a Strep tag (SAWRHPQFGGH) in the functional mutants A_{308}His2 and D_{347}His2 S318I. The D_{347}His2 S318I phage tolerated all three extended inserts, but each variant made medium-size plaques, smaller and clearer than those of f1. Interestingly, all nine of the A_{308}His5 mutants sequenced had acquired secondary mutations. Seven contained the same S318I change that improved the function of D_{347}His2 and made large plaques on K38. One (A320V; C-5241→T) was mutant two residues away from S318I, and another was more distant (S236L; C-4989→T).

To test explicitly whether S318I was responsible for the improved growth of the insertion phages, it was removed from a large plaque-forming phage (A_{308}His5 S318I) and was introduced into a small, clear plaque-forming phage (A_{308}His2). A_{308}His5 phage (without S318I) made tiny plaques on K38, while A_{308}His2 S318I now formed large turbid plaques like those of wild-type f1. These two results indicate that S318I is responsible for the improved pIV function. The S318I change also improved functioning of A_{308}His7 and A_{308}Strep-tag; these phage grew as well as wild-type f1. Plaques of the single S318I mutant were normal. Hereafter, a superscript (e.g., pIV^{Stm}; Stm stands for stabilizes mutants) denotes the presence of S318I.

Immunoblot analysis detects two forms of pIV. Western blots probed with pIV antiserum showed two reactive species in samples from phage-infected cells. One migrated at the expected position for the 45-kDa pIV monomer (Fig. 2, small arrow); the other, a large amount of reactive material in the stacking gel (large arrowhead), had not been seen previously. Samples containing either pIV^{+} (lanes 3 and 4) or pIV^{Stm} A_{308}His7 (lanes 5 and 6) gave rise to both species. The two species appeared concomitantly, beginning as early as 6 min after infection (data not shown). Uninfected cells (lane 9) or cells infected by gene *IV* deletion phage R484 (lanes 7 and 8) made neither. A gene *IV* amber mutant (R17) grown in a *supF* host made both species, but during infection of K38, which is sup^0, neither was made (not shown). Correspondingly less of each species was made by R534 (lanes 1 and 2), which synthesizes 1/10 of the amount of wild-type pIV as f1 does (38). Cells expressing gene IV^{+} from plasmid pNL200 contained both forms (lane 14), and uninduced cells produced much less of each (lane 13), while vector-containing cells produced neither (lane 12). Likewise, cells expressing gene *IV* of Ike (an f1-related phage) from a plasmid made both a 40-kDa pIV^{Ike} and a slowly migrating form (not shown).

Interestingly, appearance of the slowly migrating species required pIV export from the cytoplasm because mutant R482, whose pIV has a disabled signal sequence and is not exported (2), made only the ≈45-kDa species (lanes 10 and 11). The presence of the slowly migrating form appears to correlate with pIV function. Assembly-proficient phage making pIV^{Stm} A_{308}His7 (lanes 15 and 16) or pIV^{Stm} A_{308}Strep-tag (lanes 19 and 20) made both forms, while two less functional mutants, pIV^{Stm} D_{347}His7 (lanes 17 and 18) and pIV^{Stm} D_{347}Strep-tag (lanes 21 and 22), produced primarily 45-kDa pIV.

Results the same as those shown in Fig. 2 were obtained by the alkaline phosphase detection method. In addition, a non-antibody detection reagent (an alkaline phosphatase-core streptavidin conjugate) recognized the higher-mass species made by pIV^{Stm} A_{308}Strep-tag but not any large protein from uninfected cells, cells making pIV^{+}, or cells making pIV^{Stm} D_{347}Strep-tag, consistent with results in Fig. 2.

Together, these results demonstrate that gene *IV* specifies both the 45-kDa mono-pIV and the slowly migrating HMM-pIV.

Glycerol and urea preserve HMM-pIV during heating in SDS. It is not unusual for outer membrane proteins to resist dissociation in hot SDS (12, 28), and some integral membrane proteins aggregate during heating in SB (14, 15, 23, 45). Since pIV is an integral outer membrane protein, HMM-pIV could be an SDS-resistant species or the result of heat- or SB-induced aggregation. The possibility that heat-induced aggregation was responsible was ruled out by comparing pIV^{+}, pIV^{Stm} A_{308}His7 and pIV^{Stm} D_{347}His7 solubilized from cell membranes in SB at 4 to 100°C. Unheated samples of pIV^{+} contained much less mono-pIV than those heated to 100°C (not shown), suggesting that incubation at higher temperatures increased dissociation of HMM-pIV. HMM-pIV^{Stm} D_{347}His7 was present in unheated samples and at 37°C but dissociated into monomers at temperatures as low as 50°C. In contrast, HMM-pIV^{Stm} A_{308}His7 was highly stable, being virtually unaffected by the temperature of incubation (not shown).

We suspected that the denaturant (SB) might determine whether HMM-pIV was observed because this pIV species had not been seen in earlier studies involving immunoprecipitation of isotopically labeled proteins solubilized by heating in straight 4% SDS (i.e., in SDS dissolved in H_2O). To test this idea, samples heated in SB were immunoprecipitated, and in this case HMM-pIV was observed (not shown). Figure 3A shows that both B-ME and glycerol need to be present during heating in SDS for HMM-pIV to be seen. Only traces of HMM-pIV were present in samples heated just in SDS plus glycerol (lane 4, seen in overexposures). B-ME cannot affect pIV by reduction because this protein contains no cysteines; hence, the 10% B-ME probably acts as an organic solvent.

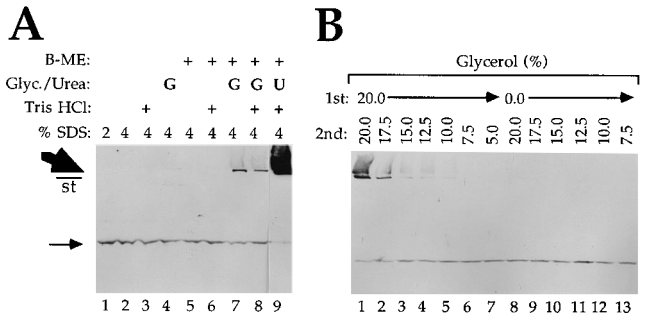

FIG. 3. Protective effects of glycerol–B-ME and urea–B-ME on the stability of HMM-pIV during heating in 4% SDS. Proteins of pIV$^+$-producing (R643-infected) cells were subjected to two-step lysis-denaturation, and the proteins were separated by SDS-PAGE and transferred to NC. Blots were probed with anti-pIV serum. Symbols are as in Fig. 2. (A) Cells were lysed by heating at 100°C (5 min) in SDS that contained the indicated constituents of SB (shown above the lanes), then mixed 1:1 with SB, and reheated (100°C, 5 min). B-ME, 10% B-ME; Glyc. or G, 20% glycerol; U, 5 M urea; Tris HCl, 125 mM Tris HCl (pH 6.8). (B) Cells were lysed by heating in SB containing either 20% or no glycerol (1st) and then reheated (2nd) in SB with glycerol at the concentrations indicated above the lanes, each time for 5 min at 100°C.

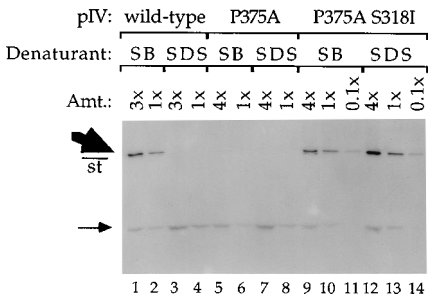

FIG. 5. The Stm (S318I) mutation stabilizes the HMM form of a multimerization-defective pIV. Replicate aliquots of pIV-producing (phage-infected) cells were heated (100°C, 5 min) in SB or 4% SDS as indicated, mixed with an equal volume of SB, and reheated (100°C, 5 min). Solubilized proteins were separated by SDS-PAGE and transferred to PVDF, and the blot was probed with anti-pIV serum. Lanes: 1 to 4, pIV$^+$ (R643); 5 to 8, pIV P375A (R616); 9 to 14, pIV^{Stm} P375A (R652). Numbers (3x, 1x, etc.) indicate the relative amounts loaded per lane. Symbols are as in Fig. 2.

HMM-pIV was also observed when SB contained 5 M urea (lane 9) or 20% sucrose (not shown) in place of the glycerol.

Figure 3B shows that HMM-pIV is preserved by glycerol–B-ME during heating in SDS. Samples heated first in standard SB (20% glycerol) and again with reduced or no additional glycerol maintained an amount of HMM-pIV reflective of the final glycerol content (lanes 1 to 7). In contrast, pIV heated first in SB lacking glycerol was devoid of HMM-pIV regardless of the final glycerol content during a second heating (lanes 8 to 13). A similar response to urea was observed (not shown). These results rule out the possibility that glycerol and urea cause HMM-pIV to form by aggregation.

HMM-pIV is the predominant form of the protein in membranes. To probe the stability of pIV during heating in SDS in the absence of stabilizing agents, total (inner plus outer) membrane samples containing pIV$^+$ were incubated in straight SDS at concentrations ranging from 0.01 to 4% at 100°C or in 4% SDS at temperatures ranging from 4 to 100°C; the solubilized portions were then analyzed. Mostly mono-pIV, and virtually no HMM-pIV, was present in samples heated at 100°C in 4, 3, 1 or 0.3% SDS (Fig. 4, lanes 1 to 4). At SDS concentrations of ≤0.1%, little or no pIV was solubilized (lanes 5 to 7). OmpA was solubilized to the same extent as pIV over this range of SDS concentrations (not shown). In contrast, HMM-pIV was the only species observed in samples solubilized with 4% SDS at or below 65°C (lanes 8 to 12). A significant portion of the pIV was in the HMM form even at 80°C (lane 13), but none remained at 100°C (lane 14). Mono-pIV was present only when samples in 4% SDS were heated above 65°C (compare lanes 8 to 12 with lanes 13 and 14). These results show that HMM-pIV is the predominant form of the protein in cell membranes. The heat modification of OmpA reflecting its unfolding (reference 12 and data not shown) and the dissociation of trimeric outer membrane porins (28) also occur only above 65°C. Thus HMM-pIV is unusually resistant to dissociation in SDS, as are these better-known outer membrane proteins.

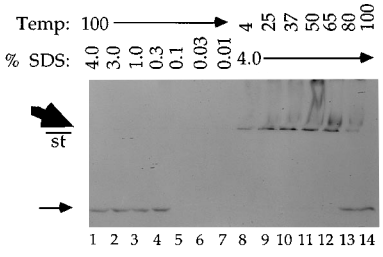

FIG. 4. Persistence of HMM-pIV in SDS as a function of temperature. Total membranes of pIV$^+$-producing (R643-infected) cells were solubilized at 100°C in the SDS concentrations indicated or in 4% SDS at the indicated temperatures (4 to 37°C, 30 min; 50 to 80°C, 15 min; 100°C, 5 min). The solubilized portions were mixed with an equal volume of SB, subjected to SDS-PAGE, and transfered to PVDF, and the blot was probed with anti-pIV serum. Symbols are as in Fig. 2.

The Stm mutation stabilizes HMM-pIV and restores *psp* induction. The pIV P375A mutant phage (R616) makes minute plaques at reduced efficiency, and the mutant protein is defective in multimerization despite its fractionation with the outer membrane (38). R652 arose as a spontaneous large plaque-forming derivative of R616 that retains the P375A mutation. The suppressor mutation that it carries, S318I (pIV^{Stm}), is the same change that improves pIV function in several His tag derivatives. The single pIV^{Stm} mutant (R672) has no discernible phenotype.

Figure 5 shows that the HMM-pIV P375A is more prone to dissociation than HMM-pIV$^+$. The double mutant pIV^{Stm} P375A has simultaneously regained function and an SDS- and heat-stable HMM form. The amounts of mono-pIV were similar for all three proteins heated to 100°C in straight 4% SDS (Fig. 5, lanes 4, 8, and 13). However, following heating in SB, the HMM form of nonfunctional pIV P375A could not be detected (lanes 5 and 6), although this form was abundant in samples of functional pIV^{Stm} P375A (lanes 9 to 11) and wild-type pIV (lanes 1 and 2). The P375A mutant protein was mostly monomeric in SB even when it was not heated (not shown). This instability could account for its poor functioning as well as our inability to detect pIV P375A multimers by cross-linking (38). By comparison, multimers of a partially functional mutant, pIV^{Stm} D_{347}His7, were readily detected by cross-linking (not shown), and its HMM form was stable in SB at intermediate temperatures, although it was less stable than HMM-pIV$^+$ (not shown).

HMM-pIV^{Stm} P375A could not be dissociated completely in straight SDS at 100°C (Fig. 5, lanes 12 to 14), whereas pIV$^+$ was (lanes 3 and 4). Remarkably, the HMM form of the singly mutant pIV^{Stm} persisted at 100°C at all conditions tested (Fig. 6). Thus, this single amino acid change confers unusual stability on the HMM form. HMM-pIV^{Stm} A_{308}His7 was also highly stable in SDS (not shown).

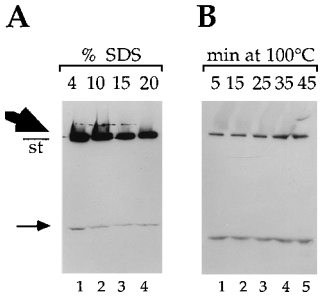

FIG. 6. Extreme heat stability of HMM-pIV^{Stm}. pIV^{Stm}-producing (R672-infected) cells were heated in the SDS concentrations and for the times indicated, mixed with an equal volume of SB, and reheated; the proteins were separated by SDS-PAGE and blotted, and blots were probed with anti-pIV serum. (A) Variable-percent SDS (100°C, 5 min, NC blot); (B) increasing times of heating (100°C, 4% SDS, PVDF blot). Symbols are as in Fig. 2.

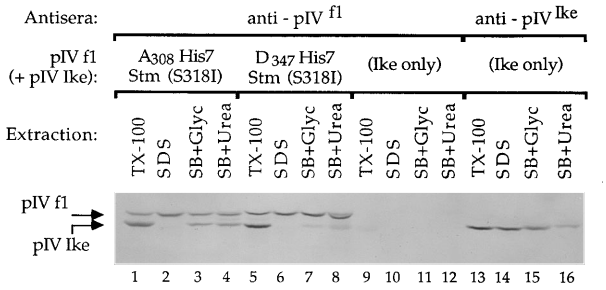

FIG. 7. SDS- and heat-stable pIV^{Ike}-pIV^{f1} heteromultimers. Cells carrying a gene *IV*^{Ike} plasmid (A627) were grown at 33°C, infected for 25 min (or mock infected), and then shifted to 38°C for 5 min to induce gene *IV*^{Ike}. Proteins were labeled for 10 min with [^{35}S]methionine, collected, processed as described below, and precipitated with either anti-pIV^{f1} or anti-pIV^{Ike} serum, as indicated above the lanes. Lanes: 1 to 4, pIV^{Ike} plus pIV^{f1,Stm} A_{308}His7 (R662); 5 to 8, pIV^{Ike} plus pIV^{f1,Stm} D_{347}His7 (R666); 9 to 16, pIV^{Ike} (uninfected). Extraction buffers and procedures: TX-100, membrane proteins were extracted at 4°C in 2% Triton X-100 (lanes 1, 5, 9, and 13); SDS, cells were heated (100°C, 5 min) in straight 4% SDS (lanes 2, 6, 10, and 14); SB+Glyc, cells were heated (100°C, 5 min) in SB (lanes 3, 7, 11, and 15); SB+Urea, cells were heated (100°C, 5 min) in SB–8 M urea (lanes 4, 8, 12, 16). After immunoprecipitation, samples were heated (100°C, 5 min) in straight 4% SDS, mixed with an equal volume SB, and reheated (100°C, 5 min), and the proteins were separated by SDS-PAGE and visualized by autoradiography. Lane 16 was loaded with half as much sample as were the other lanes. Relative amounts of pIV^{Ike} coprecipitated with pIV^{f1} by anti-pIV^{f1} were determined with a PhosphorImager and are presented with background subtracted, expressed relative to amounts precipitated by anti-pIV^{Ike} (TX-100, SDS, SB+Glyc, SB+Urea): pIV^{Ike} coprecipitated with pIV^{f1,Stm} A_{308}His7 (0.64, 0.03, 0.46, 0.66) and with pIV^{f1,Stm} D_{347}His7 (1.05, 0.06, 0.20, 0.31).

In contrast to pIV^{+}, a portion of pIV^{Stm} was monomeric when solubilized from membranes at low temperatures (≤65°C) in straight 4% SDS; however, no additional dissociation occurred at higher temperatures (not shown).

When pIV is properly localized in the *E. coli* outer membrane, it induces the *psp* operon (3). The level of PspA synthesis in cells infected with phage that either make (R643, R652, R662, R670, and R672) or fail to make (R666, R616, and R671) an SDS- and heat-stable HMM-pIV was determined by pulse-labeling and immunoprecipitation. Table 3 shows that *pspA* was induced only by phage that make SDS- and heat-stable HMM-pIV. Thus, both pIV function and *psp* induction correlate with the stability of the HMM form.

HMM-pIV is the pIV multimer. Heteromultimers composed of pIV made by phage f1 (pIV^{f1}) and pIV of phage Ike (pIV^{Ike}) are detectable by coimmunoprecipitation of native proteins (in Triton X-100 extracts) if both proteins are made in the same cell (21). To test if pIV^{f1}-pIV^{Ike} heteromultimers are SDS and heat stable, cells expressing gene *IV*^{Ike} from a plasmid were infected with phage producing pIV^{f1,Stm} A_{308}His7 (R662) or pIV^{f1,Stm} D_{347}His7 (R666) or were not infected and then were labeled for 10 min with [^{35}S]methionine. The cells were collected and heated to 100°C in straight SDS, SB, or SB plus urea. A fourth aliquot was fractionated to obtain the cell envelopes, from which membrane proteins were extracted in Triton X-100 as had been done previously (21). A portion of each sample was immunoprecipitated with antibody either to pIV^{f1} or to pIV^{Ike}. Subsequently the precipitates were heated to 100°C in straight SDS to dissociate all pIV species into monomers. Monomeric pIV^{f1} (45 kDa) and pIV^{Ike} (40 kDa) can be distinguished on protein gels by their different sizes.

Figure 7 shows that a significant amount of pIV^{Ike} was precipitated along with pIV^{f1} by the antiserum to pIV^{f1} when the coexpressing cells were heated to 100°C in SB or in SB plus urea (lanes 3, 4, 7, and 8). In contrast, pIV^{Ike} was not coprecipitated if cells were first heated to 100°C in straight SDS (lanes 2 and 6). This result indicates that pIV^{f1} and pIV^{Ike} form heteromultimers that are resistant to SDS if they are heated in the presence of glycerol–B-ME or urea–B-ME. Somewhat more pIV^{Ike} was coprecipitated following heating in SB plus urea than in SB (compare lanes 4 and 8 with lanes 3 and 7). This effect parallels the greater stabilizing effect of urea than of glycerol on HMM-pIV seen by immunoblot analysis (Fig. 3A). Less pIV^{Ike} was coprecipitated with pIV^{f1,Stm} D_{347}His7 than with pIV^{f1,Stm} A_{308}His7 after heating in SB or SB plus urea (compare lanes 3 and 7 and lanes 4 and 8). This result presumably reflects reduced heat stability of pIV^{Ike}-pIV^{f1,Stm} D_{347}His7 heterocomplexes, consistent with denaturation data presented in Fig. 2. Comparable results were obtained when the stability of heterocomplexes was assessed by determining how much pIV^{f1} was coprecipitated with pIV^{Ike} by antiserum to pIV^{Ike} (not shown).

Consistent with previous findings (21), no heterocomplexes were obtained when cells making only pIV^{f1,Stm} were mixed with an equal number of cells making only pIV^{Ike} and then heated together (to 100°C in SDS, SB, or SB plus urea), nor were they seen when native extracts were mixed. This finding demonstrates that the Stm mutation does not promote aggregation of isolated pIV subunits and thus confirms that the heterocomplexes form in vivo and not after extraction. Thus,

TABLE 3. Correlation of PspA synthesis, SDS stability, and heat stability of HMM-pIV and pIV function

Phage	pIV	PspA[a]	HMM-pIV[b]	Plaque size
None	None	0.03	NA[c]	NA
R643	Wild type	1.00	+	Large
R616	P375A	0.01	−	Minute
R652	P375A S318I (Stm)	1.34	+	Large
R662	A_{308}His7 S318I (Stm)	1.09	+	Large
R666	D_{347}His7 S318I (Stm)	0.06	−	Small
R670	A_{308}Strep-tag S318I (Stm)	1.15	+	Large
R671	D_{347}Strep-tag S318I (Stm)	0.04	−	Small
R672	S318I (Stm)	1.26	+	Large

[a] K38 cells grown in minimal medium and infected with phage for 25 min or mock infected were labeled for 1 min with [^{35}S]methionine. Samples were precipitated with trichloroacetic acid, solubilized in SDS, immunoprecipitated with PspA antiserum and staphylococcal protein A-Sepharose, and then fractionated by SDS-PAGE (15% polyacrylamide gel), and the PspA bands were quantified with a PhosphorImager.

[b] Symbols: +, abundant HMM-pIV in samples heated to 100°C in SB; −, HMM-pIV was absent or present in only trace amounts following this treatment.

[c] NA, not applicable.

the coimmunoprecipitation data establish a link identifying HMM-pIV as the pIV multimer.

DISCUSSION

We have previously used three methods to demonstrate the multimeric nature of pIV: velocity sedimentation, chemical cross-linking, and coimmunoprecipitation of pIV homologs (21, 38, 42). Here we report a simpler way to detect multimers: the pIV multimer appears as a slowly migrating, HMM form in the stacking gel during denaturing PAGE. This gel assay method allows a fast and easy comparison of the relative stabilities of wild-type and mutant pIV complexes.

Our first objective was to establish whether HMM-pIV originates from in vivo multimeric pIV. As is true for the authentic pIV multimer (21), pIV is the only phage gene product required to form HMM-pIV. Like porins (49), pIV must be exposed to outer membrane constituents in order to multimerize (21). In accordance with this finding, the nonexported pIV signal sequence mutant is not in the HMM form, just as it fails to form mixed multimers with wild-type pIV^{Ike} (21).

Some membrane proteins exhibit unusual mobilities during SDS-PAGE after heating in SDS. For this reason, we went to some length to demonstrate that HMM-pIV is derived from the multimer and is not simply a gel or heating artifact. From this analysis, we have learned that the pIV multimer is stable in 4% SDS at ≤65°C and that it remains largely associated even at 100°C in the presence of stabilizing agents (B-ME and glycerol, urea, or sucrose); however, heat-induced aggregation is not observed. The failure of the multimer of pIV to enter standard SDS-polyacrylamide gels can be explained by exclusion due to its mass, which was determined from sedimentation analysis to be ≈500,000 Da (21). In contrast, undissociated porin trimers are small enough (≈100 kDa) to migrate normally on polyacrylamide gels (49). The anti-pIV serum used here recognizes both native and SDS-dissociated pIV. Thus, it is not known whether undissociated pIV in stacking gels retains native epitopes. It is unlikely that the HMM-pIV complex contains protein species other than pIV, since earlier studies found no evidence for either coimmunoprecipitable protein species or cross-linking partners of pIV, aside from pIV itself (21, 38).

An important conclusion of this analysis is that essentially all of the pIV in membranes is multimeric, which indicates that a pool of pIV monomers does not usually exist in the outer membrane. We have previously reported that a variable fraction of pIV in Triton X-100 extracts sediments at 18S and the remainder is found at the top of sucrose gradients (21). The nonsedimenting pIV could have reflected the existence of a substantial monomer pool. However, the observation that virtually all pIV is in the HMM (multimeric) form indicates that the nonsedimenting material cannot be monomer. This conclusion is supported by additional experiments that show that nonsedimenting pIV can still be cross-linked and that a substantial fraction of pIV^{f1}-pIV^{Ike} complexes remain at the top of gradients (41). The nonsedimenting pIV may be multimers that remain associated with lipid.

The abundance and relative SDS and heat stabilities of the HMM forms of mutant pIVs correlate well with their levels of function. This finding suggests that the multimeric form of pIV reflects the structure that actually participates in phage assembly-export. The clearest example of this is pIV P375A, which displays dramatic increases in the amount and stability of its HMM form concurrently with a gain of function due to the second-site suppressor mutation, Stm (S318I). This is the first direct demonstration that the multimer of pIV, or of any homolog, is required for export function. The observations that pIV^{Ike} has an HMM form and that pIV^{f1}-pIV^{Ike} complexes are also SDS and heat stable substantiate the link between the HMM form and multimeric pIV.

To date, one pIV homolog has been shown to form a homomultimer. Like pIV, the *N. gonorrhoeae* OMP-MC protein (now renamed PilQ [7]) forms a large complex of ≈10 to 12 subunits that migrates slowly in polyacrylamide gels (7, 30). Both reduction and alkylation are needed to dissociate the PilQ complex into monomers (30), whereas neither is needed to disrupt the pIV multimer; this could be due to the many cysteine residues in PilQ (absent in pIV) that could be disulfide bonded (29, 30).

In addition to pIV and PilQ, evidence is accumulating that other homologs may form multimers. Some form mixed multimers with pIV^{f1} (21). Cross-linking and gel filtration were used to show that XpsD of *Xanthomonas campestris* pv. campestris is part of a large complex which, if it is composed exclusively of XpsD, should contain ≈12 subunits (5). The homologs YscC of *Y. pestis* (32) and PulD of *Klebsiella oxytoca* (17) were recently reported to exhibit dual electrophoretic migration in protein gels, consistent with multimer formation. It seems likely that multimer formation is a general property of this diverse family of outer membrane proteins.

One advantage of the phage system is the ease with which improved strains can be selected as a result of rapid rates of viral evolution. f1 multiplication is a process normally balanced between component synthesis and phage export, and situations (genetic or environmental) that slow assembly-export result in the premature death of infected cells and low phage yields. As a result, viral mutations that reverse an imbalance (i.e., that improve assembly-export and increase phage yields) are strongly selected for. The Stm (S318I) mutation was isolated as a second-site suppressor in three gene *IV* mutant strains that were subjected to challenging growth conditions. It is likely that Stm is an example of global suppression, wherein different mutant alleles at diverse positions can all be corrected by the same second-site amino acid change, as was described for staphylococcal nuclease (50) and the phage P22 tail spike protein (9).

The extreme resistance of the Stm single mutant to dissociation in SDS suggests that this mutation minimizes the energy needed to achieve or maintain the mature, folded form of the protein. The extent to which Stm restores function to different pIV mutants (P375A ≥ A_{308}His7 > D_{347}His7) may correlate with the degree of structural distortion induced by the original mutation. It is noteworthy that a fraction of pIV^{Stm} is monomeric in SDS at all temperatures. This finding suggests that the mutation is also responsible for a small number of misfolded subunits, which could be unable to multimerize, or for occasional misalignment of subunits, which could result in multimers that are more easily dissociated. Residue 318 may define a site where intra- or intersubunit hydrogen bonding by serine has been replaced in the mutant by a hydrophobic interaction involving isoleucine. Isoleucine is found at this position in about half of the pIV homologs in the alignment reported by Genin and Boucher (13).

The Stm mutation also increases the steady-state level of pIV P375A in cells. This is due to reduced rates of degradation which may result from more efficient multimer formation, since gene *IV* expression measured in a pulse-labeling is not affected (not shown). The mutant pIVs, and presumably wild-type pIV as well, could be protected against proteolysis by forming multimers, in much the same way that subunits in heterologous complexes can be stabilized by their mutual in-

teractions (e.g., stabilization of SecY by SecE [51] and of TonB by ExbB [1, 11]).

The Stm mutation was not found as a second-site suppressor of pIV mutants N_{269}His2 and Q_{336}His2, which appear to interfere with wild-type pIV function. The dominant-negative interference phenotype is thought to result from the formation of stable but nonfunctional mixed multimers composed of both mutant and wild-type pIVs. In keeping with this interpretation, the N_{269}His2 and Q_{336}His2 mutant proteins were stable during pulse-chase labeling experiments (not shown). If insertions at N-269 and Q-336 decrease the internal dimensions of the hypothetical assembly-export channel, entry into or egress from the channel could be hindered, as suggested before for other interfering mutants (38).

Little is known about the structure of the pIV subunit or multimer (or of any of the pIV homologs). Since pIV is not a member of the porin family, the crystal structures of OmpF, PhoE, and LamB of *E. coli* (6, 47) and of *Rhodobacter* porins (22) have been of limited use in developing a conjectural model of pIV. However, the fact that insertions at four of the five sites in pIV where β-turns were predicted preserved overall the conformation of pIV, as revealed by assembly proficiency (A-308 and D-347) or by interference (N-269 and Q-336), provides an encouraging start toward understanding the structure of this protein. More detailed information about pIV structure and function can be anticipated from studies of the native, multimeric complex.

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