Biochemical and Genetic Evidence for Three Transmembrane Domains in the Class I Holin, \(\lambda\) S

Angelika Gründling‡§, Udo Bläsi§, and Ry Young‡¶

From the ‡Department of Biochemistry and Biophysics Texas A&M University, College Station, Texas 77843-2128 and the §Institute of Microbiology and Genetics, Vienna Biocenter, University of Vienna, Dr. Bohrgasse 9, 1030 Vienna, Austria

\(\lambda\) S, the prototype class I holin gene, encodes three potential transmembrane domains in its 107 codons, whereas 21 \(\lambda\) S, the class II prototype spans only 71 codons and encodes two transmembrane domains. Many holin genes, including \(\lambda\) S and 21 \(\lambda\) S, have the “dual-start” regulatory motif at the N terminus, suggesting that class I and II holins have the same topology. The primary structure of 21 \(\lambda\) S strongly suggests a bitopic “helical-hairpin” topology, with N and C termini on the cytoplasmic side of the membrane. However, \(\lambda\) S chimeras with an N-terminal signal sequence show Lep-dependent function, indicating that the N-terminal domain of \(\lambda\) S requires export. Here the signal sequence chimera is shown to be sensitive to the missense change A52V, which blocks the marker which has been extensively studied for the \(\lambda\) holin. Lysis normally occurs 50 min after induction of a \(\lambda\) lysogen, but missense mutants of \(\lambda\) S are known which exhibit lysis times from 19 min to greater than 2 h (6, 7). Moreover, lysis can be triggered prematurely by addition of an energy poison, like cyanide, to the medium (8). Deletion of the C-terminal charged domain of \(\lambda\) S significantly reduces the ability of \(\lambda\) to lyse, whereas replacement of the normal sequence with a heterologous sequence with more positive charge retards lysis (5). A remarkable feature of the \(\lambda\) gene, and other holin genes, is the “dual start motif”: i.e. two translational start codons, at positions 1 and 3 (Fig. 1A), which give rise to two products, called S107 and S105 (9–11), with opposing functions. That is, S105 acts as the lethal holin, while S107 acts as an inhibitor of S105 (1, 9, 11, 12). Within certain ranges, the proportion of S107 and S105, which is determined by an RNA stem-loop structure overlapping the translational initiation region (9–11) and which is normally 1:2, is an important determinant of the lysis “clock” (13) (Fig. 1B). The operational difference between S107 and S105 is the positively charged residue at position 2; replacement of this residue with a neutral or acidic residue ablates inhibitor function and converts S107 into a lethal holin (11). Structural information is limited and restricted to \(\lambda\) S, which in the detergent octyl glucoside has a CD spectrum consistent with approximately 40% \(\alpha\)-helical structure (14). The topology of \(\lambda\) S is as yet undefined. The C terminus of \(\lambda\) S has been shown to be cytoplasmic, by the criterion of protease sensitivity and resistance in inverted membrane vesicles (IMV) and spheroplasts, respectively (5). CD analysis

Host lysis for double-stranded DNA bacteriophages involves active degradation of the host peptidoglycan by enzymes designated as endolysins, or phage-encoded muranylactic activities (1). Diverse enzymatic activities fulfill this role in different phages but a common feature is that, despite the fact that their substrate is outside the cytoplasmic membrane, endolysins have neither a secretory signal sequence nor a specialized secretory system. Instead, endolysins require the function of a membrane protein called a holin to exit the cytosol and gain access to the cell wall. Active endolysin accumulates in the cytosol during the vegetative cycle until, at a genetically programmed time, the holin proteins somehow permeabilize the membrane and allow escape of the endolysin. Holins thus control the timing of host lysis and the progeny yield of the infective cycle. Nothing is known about the nature of the “hole,” the membrane lesion produced by the holin, except that it is apparently nonspecific in that different holins can function with different endolysins.

1 The abbreviations used are: TM, transmembrane helix; CD, circular dichroism; DTT, dithiothreitol; IASD, 4-acetamido-4-((ido-acyethyl)amino)stilbene-2,2’-disulfonic acid, disodium salt; IMV, inverted membrane vesicles; PAGE, polyacylamide gel electrophoresis; TBS, Tris-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxy-methyl)-ethyl]glycine.

* This work was supported by United States Public Health Service Grant GM27099 and funds from the Robert A. Welch Foundation and Texas Agricultural Experiment Station. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 409-845-2087; Fax: 409-862-4718; E-mail: RYLAND@TAMU.EDU.

This paper is available on line at http://www.jbc.org

Vol. 275, No. 2, Issue of January 14, pp. 769–776, 2000
Printed in U.S.A.
detergent-solubilized S indicates that about 40–45 residues are in helical conformation, suggesting two TM helices, assuming the detergent environment preserves the secondary structure (14). The distal hydrophobic domain (residues 65–83) has a proportion of hydroxylated residues which would be unusual for a TM domain. These data suggested a model with hydrophobic domains 1 and 2 acting as TM domains (Fig. 1C, model 3). However, gene fusion studies in which a secretory signal sequence was fused to S indicated that the N terminus must be externalized for lysis to occur, suggesting a topology with 3 TM domains (Fig. 1C, model 5) (15). In contrast, the topology of S21 is to be evident from inspection of the primary structure, with the two uniformly hydrophobic domains of the right length for TM helices and the C terminus rich in basic residues, strongly suggesting a “C-inside, N-inside” orientation (Fig. 1C, model 4).

Recently, it has been demonstrated that S21 has a dual start motif functionally homologous to S, giving rise to two S21 protein products of 71 (S21a) and 68 (S21b) amino acids that function as inhibitor and effector, respectively (16) (Fig. 1A and B). Phylogenetic analysis shows that many holin genes, of both classes, appear to have a dual start motif. Thus the simplest expectation is that the dual start motif in class I and II holins should function from the same side of the membrane, a notion which would support models with two TM domains for λ S (Fig. 1, model 3).

Here we report a cysteine-scanning analysis of the S gene, which has only a single-Cys codon in the parental allele (Fig. 1A). The properties of the mutant alleles are examined and the accessibility of the Cys residues of the membrane-embedded protein products is determined by chemical modification. The results allow an unambiguous determination of the membrane topology of S, and by extension, all class I holins.

**EXPERIMENTAL PROCEDURES**

**Strains, Bacteriophages, Plasmids, and Growth Media**—The strains MC4100, XLI-Blue, and pop2135, the lysis-defective thermostable phase AΔSR and the parental plasmid pS105 have been described (14, 17). Media, growth conditions, and thermo-induction have been described (14). Cells carrying plasmids were grown in LB-ampicillin (LB supplemented with 100 μg/ml ampicillin).

**Standard DNA Manipulation, Polymerase Chain Reaction, Site-directed Mutagenesis, and DNA Sequencing**—These methods have been described previously (14, 18). Site-directed mutagenesis was performed using pairs of synthetic oligonucleotides purchased from the Gene Technology Laboratory of the Department of Biology, Texas A&M University. In each case, 17–30 nucleotides of the parental sequence flanked the 1–3 nucleotides mutated sequence. All constructs were checked by automated fluorescent sequencing (14). For the construction of the AΔSR alleles of the VIIΔS fusions, the pl vector plasmids pVIII-S105 or pVIII-S107 (15) were used as templates, and the products were designated pVIII-S105ASN and pVIII-S107ASN, respectively.

**Labeling of IMV or Whole Cells with 5 mM IASD—**IMV, purchased from Molecular Probes (Eugene, OR), was freshly dissolved in TBS buffer, pH 7.2, to a final concentration of 100 μM to 200 μM of IMV or whole cell samples were labeled with 5 mM IASD for the indicated time in the dark. The reactions were stopped with 0.15 M cysteine and incubation for 15 min at room temperature. A stock solution of 1 mM cysteine was prepared just prior to use in TBS. As a negative control for labeling, the cysteine was added to one sample before addition of IASD. IMV were collected by ultracentrifugation at 100,000 × g for 1 h at 18 °C. Whole cells were disrupted by sonication in a cup horn attachment of a sonicator model W-375 (Heat Systems, Ultrasonics, Plainview, NY). Cells were sonicated 6 × 30 s in the continuous mode and kept on ice for at least 1 min between the cycles. Following sonication, the cell debris were collected by ultracentrifugation. Membrane pellets were solubilized in 50 μl of membrane extraction buffer (1% Triton X-100, 10% glycerol, 0.5 mM NaCl, 35 mM MgCl2, 20 mM Tris-HCl, pH 8.0) supplemented with 0.2 mM cysteine for 6 to 8 h at 37 °C with shaking (19, 20). Afterward detergent-insoluble material was washed twice with 100,000 × g for 45 min at 18 °C to produce a detergent-solubilized preparation of inner membrane proteins. For SDS-PAGE electrophoresis the detergent soluble fraction were diluted 1:1 with 2× protein sample buffer containing 2.8 M β-mercaptoethanol (13). Protein samples were boiled for 5 min and centrifuged at 14,000 × g for 5 min at room temperature just before loading on an SDS-PAGE gel.

**Labeling under Denaturing Conditions to 2% SDS and 150 mM IASD at 100 °C—**IMV were prepared as described above, with the exception that the cell pellet of the induced culture was resuspended in 1 ml of TBS supplemented with 0.5 mM DTT. A 100 mM stock solution of DTT was prepared in TBS just prior to use. 50 to 200 μl of IMV were collected by ultracentrifugation at 100,000 × g for 1 h at 18 °C. The membrane pellet was solubilized in 50 μl of 2% SDS, 0.5 mM DTT, TBS buffer. Membrane proteins were extracted for 6 to 8 h at 37 °C with shaking. Insoluble material was removed by ultracentrifugation. Shortly before labeling reaction, 0.5 mM DTT was added and the samples were kept for 10 min at room temperature. For the labeling reaction under denaturing conditions, 15 mM IASD was added from a 100 mM stock solution dissolved in TBS buffer and samples were incubated for 15 min at 100 °C. The reactions were stopped with 0.4 mM cysteine from a freshly prepared 1× stock solution in TBS buffer and incubated for 15 min at room temperature. Protein samples were mixed with the same volume of 2× protein sample buffer containing 2.8 M β-mercaptoethanol. The samples were boiled for 5 min at 100 °C, centrifuged for 5 min at 14,000 × g at room temperature, and loaded on the SDS gel.

**Labeling of IMV with 15 mM IASD in High Salt Conditions—**20 μl of induced culture were resuspended in 1 ml of TBS buffer (0.75 mM NaCl, 25 mM Tris, pH 8.0), supplemented with 1 mM DTT. The labeling reaction was performed for 60 min at room temperature with 15 mM IASD, dissolved as a 100 mM stock solution in TBS buffer (TBS buffer, pH 8.0). Reactions were stopped with 0.4 mM cysteine and inner membrane protein extracts were prepared for SDS-PAGE and Western blot analysis as described above.
SDS-PAGE, Western Blotting, and Immunodetection—For immuno-
detection of S protein, protein samples and prestained molecular mass
standards (Life Technologies, Inc., Gaithersberg, MD) were resolved on
16% Tris/Tricine gels. Tris/Tricine 16% SDS-PAGE was performed ac-
cording to the method of Schägger and Jagow (21). Proteins were then
transferred to a 0.1-
\( \mu \)m nitrocellulose membrane (Schleicher & Schuell)
with a semi-dry blotting apparatus from Enprotech (Natick, MA) for 2
to 3 h at 100 mA. Western blots were developed as described by Towbin
et al. (22). An antibody raised in rabbits against a synthetic C-terminal
peptide from the S sequence (13) was used as primary antibody at a
dilution of 1:1,000. The secondary antibody (goat anti-rabbit immuno-
globulin G conjugated to horseradish peroxidase) was purchased from
Pierce and used at a dilution of 1:1,000. Blots were developed according
to the manufacturer’s instructions. Images of blots were digitized with
a Hewlett-Packard ScanJet IIcx scanner (Palo Alto CA). Quantitative
analysis of immunoblots was performed using the image analysis pro-
gram NIH image (version 1.54) public domain software suit (W. Ras-
band, National Institutes of Health). The labeling efficiency of the
single cysteines was calculated with the formula,
\[
\% \text{ Labeling} = 100\% \times \frac{\text{Intensity of modified band}}{\text{Intensity of modified band + intensity of unmodified band}}
\] (Eq. 1)

RESULTS

Signal Sequence-dependent Lysis Abolished by Holin De-
fect—A common feature of the inhibitor and putative inhibitor
products of holin genes with dual start motifs is that there is at
least one positively charged residue in the N-terminal exten-
sion (Fig. 1B). The ability of the S107 product of \( \lambda S \) to exert its
inhibitory function is abolished by energy poisons (11). More-
over, the inhibitor form alone can be triggered to cause lysis by
cyanide, unless two or more positively charged residues are
present at the N terminus (11, 23). These observations sug-
gested that the N terminus of both S products might require
transit of the membrane, after more distal segments of the
protein have integrated into the bilayer (Fig. 1C, model 1
and 2, and 3). In model 2, the putative TM domains are numbered,
as are the putative loops (L1 and L2). Model 4 depicts the presumed topology for 21 S, and model 5 shows the leader peptidase (Lep) cleavage site
for the VIII-S fusion protein (15) and its presumed topology.

Fig. 1. Primary structure, dual start motif, and proposed topology for the class I holin (\( S^+ \)) and class II holin (\( S^\prime \)). A, the predicted
primary structures of \( \lambda S \) and \( 21 S \) are shown. Charged residues (\( \pm \)), putative TMs (\( \text{**} \)), turn regions (ttt), and highly charged C-terminal regions
(*** ) are indicated in the sequences. # indicates the dual starts of \( \lambda S \) and \( 21 S \) (16, 35). Shadowed boxes indicate the protected core regions of the
TM helices identified in this study. B, the dual start motifs of \( \lambda S \) and \( 21 S \) are shown. The boxed sequences indicate the Shine-Dalgarno sequences
for the dual translational start of the S genes. The product sizes in amino acid residues are given in parentheses for both holins. C, topological
models for \( \lambda S \) shown with two or three \( \alpha \)-helical transmembrane domains (models 1, 2, and 3). In model 2, the putative TM domains are numbered,
as are the putative loops (L1 and L2). Model 4 depicts the presumed topology for 21 S, and model 5 shows the leader peptidase (Lep) cleavage site
for the VIII-S fusion protein (15) and its presumed topology.

Three Transmembrane Domains for \( \lambda S \)
Three Transmembrane Domains for λ S

Fig. 2. The A52V substitution blocks lysis caused by the VIII-S chimera but not its accumulation in the membranes. A. pop2135 cells harboring plasmids with VIII&S fusion genes were thermally induced at time 0 and monitored for culture mass as A550. Plasmids: pVIII-S105, induced (●) or uninduced (▲); pVIII-S105ASCV, induced (○), pVIII-S107, induced (■), pVIII-S107ASCV, induced (□). To check for endolysin production, CHCl3 was added (vertical arrow) to a portion of the induced pVIII-S105ASCV and pVIII-S107ASCV cultures (dashed lines). B. Western blot analysis of the accumulation of the hybrid protein in the membrane. Inner membrane protein samples were prepared from induced and uninduced cultures. Samples of induced cultures were taken after cell lysis was completed, or, for cultures which did not undergo lysis, at 20 min after induction. Samples from the uninduced cultures were taken at 20 min after induction. Lane 1, prestained molecular weight marker; lanes 2–5, uninduced cultures; lanes 6–9, induced cultures; cells carrying pVIII-S105 (lanes 2 and 6), pVIII-S107 (lanes 3 and 7), pVIII-S105ASCV (lanes 4 and 8), and pVIII-S107ASCV (lanes 5 and 9). Arrows indicate positions of S monomers and dimers. Numbers to the left of the panel are the sizes of molecular mass standards in kDa.

Fig. 3. Labeling of single-Cys S proteins in IMV reveals protected regions. A. IMV containing S proteins were treated with 5 mM IASD for 60 min and analyzed by SDS-PAGE and Western blot. Modification of each single-Cys S protein was seen as reduced SDS-PAGE mobility. Representative single-Cys S proteins are shown. For each sample, the + lane is the treated sample and the – lane represents a control reaction where the exogenous blocking cysteine is added before the IASD reagent. Molecular mass standards of 14.6 and 5.8 kDa are shown in lanes labeled “mw.” B, graphical representation of labeling efficiency. Positions of the single Cys in the parental sequence, plus each of the 22 Cys substitutions within S, and also the position of the Cys appended to the C terminus (r108), are indicated on the x axis.

Support the idea that S imbeds in the membrane with three TM domains in an N-out, C-in topology (Fig. 1C, model 2).

Chemical Probing of S Topology—The parental S sequence has a single-Cys codon at position 51 (Fig. 1A). The C51S allele is fully lytic, and a large collection of alleles with single-Cys substitutions have been created, all of which generate stable, membrane-inserted proteins and most of which retain lytic capacity. To probe the topology of S in the membrane, each of these alleles was expressed in bacterial cells and IMV were prepared from each induced culture. These samples were treated with IASD, a cysteine-specific modification reagent, and the extent of modification of the different single-cysteine S proteins assessed by monitoring SDS-PAGE mobility (Fig. 3). Three hydrophobic regions of the S sequence were resistant to modification, including residues 23–26, 49–54, and 74–81. These clusters of resistant regions map to the core of predicted TM domains in the 3 TM model (Fig. 3). The chemical reactivity of these sites was confirmed by treating the samples with boiling SDS to solubilize the S protein (Fig. 4). These results strongly suggest that S has three membrane-imbedded domains centered on these protected regions.

The 3 TM model would predict that there would be 4 aqueous domains (N terminus and loop 2 in the periplasm, loop 1 and C terminus in the cytoplasm). Given the membrane-impermeant nature of IASD, the simplest expectation would be that the N terminus and loop 2 would be sensitive in spheroplasts and resistant in IMV, whereas the C terminus and loop 1 would show a complementary pattern. However, multiple sites in the
N terminus and both putative inter-TM loops were sensitive in IMV (Fig. 3). Previous studies with these IMV preparations showed that at least 95% of the vesicles are inverted, as judged by the accessibility of the cytosolic C terminus of S to proteases (5). Thus, the simplest explanation for the labeling on both sides of the membrane is that the “hole-forming” ability of S makes the membrane permeable to IASD. In this case, the requirement for the reagent to pass through the S-mediated lesions might impose a kinetic difference in the modification reaction, as observed for cis and trans sites within the pore-forming toxin of *Saccharomyces aureus* (27). Fig. 5 shows that, as predicted, sites in the N-terminal domain and in loop 2 are more efficiently modified in spheroplasts, whereas a site in loop 1 is better labeled in IMV, again supporting a 3 TM model with N-out and C-in (Fig. 1C). Interestingly, the ability to label on either side of the membrane, albeit with different efficiencies, persists even with the inclusion of the A52V mutation. This indicates, that even a non-lytic S protein confers permeability defects, at least for the IASD reagent.

The most unexpected result was that several sites in the C-terminal domain, which has been demonstrated to reside in the cytoplasmic compartment by protease-accessibility studies (5), were resistant to modification in vesicles (Fig. 3). The C-terminal region is rich in charged residues, especially basic residues, and is non-essential for S function (5, 28). Moreover, replacing the C-terminal domain with unrelated sequences of comparable size but increased positive charge results in a dominant-negative phenotype (5, 28). We reasoned that these properties and the IASD resistance of the C-terminal sites in vesicles might reflect an intimate association of the C-terminal domain with the negatively charged inner surface of the cytoplasmic membrane. To test this idea, IASD modification was repeated on membrane vesicles in the presence of high salt to disrupt the putative electrostatic interactions. Under the high salt conditions, the C-terminal sites (positions 94 and 95), but not a site in the core of the putative TM3 (position 78), are quantitatively modified in IMV (Fig. 6), strongly supporting the idea that the C terminus is located on the cytoplasmic side of the bilayer and probably intimately associated with the membrane surface by virtue of ionic interactions.

**An Oligomerization Defect Has No Effect on the Pattern of IASD Modification**—S has been shown to oligomerize in the membrane (3, 29, 30). A possible alternative interpretation of the resistance of the three hydrophobic domains to IASD modification is that intimate protein-protein contacts, rather than the impermeant core of the bilayer, block access of the reagent to some of the sites. Considering that the resistance spans one or more entire helical turns of the putative TM domains and the fact that S is such a small protein, such extensive protein-protein contacts would require an oligomeric structure. As noted above, the A52V substitution abolishes S lytic function and cross-linking studies have demonstrated that this defect is associated with a failure to oligomerize. Fig. 7 shows that the pattern of IASD modification is unaffected by the A52V substitution. This indicates that the patterns of resistance within the hydrophobic domains reflect sequestration in the core of the bilayer rather than within an oligomeric assemblage, and that there is no gross alteration of topology concomitant with the lytic function of S.

**DISCUSSION**

**The Topology of Class I Holins**—The topology of λ S, the prototype class I holin, is ambiguous from examination of the primary structure. Although there are three domains with net neutral charge, only the putative TM1 and TM2 domains have a preponderance of hydrophobic residues, and TM3 is unusually rich in hydroxylated amino acids for a TM domain. Clusters of mutations with lysis-defective phenotypes involving changes in the size of side chains have been found in both TM1 and TM2, but not TM3. CD spectroscopy of purified, detergent-solubilized S supports a 2 TM model (14). Moreover, elementary primary structure constraints (*i.e.* approximately 20 amino acid residues per TM helix) make it highly likely that S21, the prototype class II holin, has only two TM domains (Fig. 1, A and C). If for no other reason than intellectual economy, the presence of functionally homologous dual start motifs in both class I and class II holins (2, 12, 16) would suggest that the N-terminal domain of both classes of holins are on the same side of the membrane; *i.e.* the cytoplasmic side. However, the results presented here constitute convincing evidence for a model in which the S holin of phage λ is integrated in the membrane with 3 TM domains, with N-out and C-in (Fig. 1C, model 2).

First, we showed that the lytic capability of a gene fusion between the signal sequence domain of M13 VII and S is sensitive to the same missense change, A52V, which blocks lysis in the native holin context. Given the conservative nature of this substitution and the specific molecular defect associated with it in the S context (*i.e.* failure to oligomerize) this strongly suggests that the presence of a cleavable signal sequence does not direct S along a fundamentally different pathway to lysis. Taken with the finding of Graschopf and Bläsi (15) that the presence of the signal sequence bypasses the block associated with the presence of positive charge at the N terminus and with the requirement for concomitant signal sequence cleavage, these results strongly indicate that the N-out, 3 TM topology for S must be considered to be at least an active form of the holin at the end of the pathway which leads to “hole-formation.”

These findings, while persuasive, do not rule out the possibility that a complete re-orientation of the S protein domain occurs after cleavage of the signal sequence in the chimeric protein, allowing S to assume a different topology. For example, after cleavage, the N terminus could in principle slip back

---

3 A. Gründling and R. Young, manuscript in preparation.
out of the bilayer and thus allow S to re-insert with a 2-TM, N-in, C-in topology (Fig. 1C, models 1 and 3). The cysteine modification studies allow us to rule out this possibility and provide convincing evidence that the central region of three potential TM domains are embedded in the bilayer. TM domains in the cytoplasmic membrane are always found to be α-helical (31). In view of the presence of a strong helical signal, and the absence of β-sheet signal, in the CD spectrum of purified S (14), one would expect each of the three regions of IASD insensitivity to extend approximately 20 residues. Using similar hydrophilic cysteine modification reagents to probe other oligotopic membrane proteins, other workers have found that only a central core of about 10 residues is protected in TM helices (32, 33). Given the asymmetry of these reagents, which,
in the case of IASD, results in a 7.5-Å separation between the charged groups conferring the hydrophilicity and the reactive group, it is likely that the reactive moieties are able to enter the bilayer to a considerable extent. Alternatively, membrane proteins, and especially S, may destabilize the bilayer locally and allow partial penetration of the reagents. In any case, it is clear that this type of methodology is capable of establishing the existence of a TM domain by mapping out the protected central core, but it is not capable of determining the boundaries of the TM and aqueous domains. For the purposes of this study, however, the existence of three protected cores clearly indicates the existence of three TM domains for this model class I holin.

It is not clear what the nature of the S hole is, but whatever it is, it was not unexpected that domains predicted to be in either aqueous compartment would be labeled by IASD treatment of membrane vesicles, because most of the single-cysteine mutants are functional as lytic proteins and thus would be expected to allow the reagent through the membrane. The difference in rate and efficiency of labeling of putative N-terminal, loop 1, and loop 2 sites in spheroplasts and inverted vesicles is consistent with this interpretation (Fig. 5). Nevertheless, in one case where the cysteine substitution abolishes S lytic function, the mutant with the S66C and A52V substitutions, the single cysteine is labeled in both spheroplasts and inverted vesicles (Fig. 5), albeit more slowly in the latter. This is difficult to rationalize, especially considering the fact that expression of these single-Cys S alleles is at the normal in vivo levels. However, it should be noted that preparation of vesicle and spheroplast samples requires time intervals which are relatively long compared with the normal kinetics of lysis after induction of a thermo-sensitive lysogen. Alleles which are defective in supporting the saltatory lysis characteristic of λ S might still yield S products which would damage the integrity of the membrane on these long time scales. It is also possible that S protein, even if blocked in the steps leading to release of the endolysin to the periplasm, may still have a significant destabilizing effect on the membrane, especially after the manipulations involving severe physical stress which are required to form IMV.

Implications for the Pathway of Holin-mediated Lysis and Its Regulation—A fundamental characteristic of holin function is that it is distinctly saltatory; that is, holins accumulate in the membrane throughout the late protein synthesis phase of the vegetative cycle, without apparent affect on host physiology, until suddenly, at a programmed time, holin function triggers, the membrane is permeabilized, host respiration stops, and the vegetative cycle, without apparent affect on host physiology, begins. As usual, the reliable clerical assistance of Shayril Pressley is gratefully acknowledged.

Acknowledgments—The guidance of Hagan Bayley in the design and implementation of the cysteine modification experiments was vital to this effort. We are also indebted to Bill Roof, David Smith, other past and present members of the Young laboratory, and Anton Graschopf for their collegial interactions and assistance. As usual, the reliable clerical assistance of Shayril Pressley is gratefully acknowledged.

REFERENCES

1. Young, R. (1992) Microbiol. Rev. 56, 430–481
2. Young, R., and Blasi, U. (1995) FEMS Microbiol. Rev. 17, 191–205
3. Smith, D. L. (1996) Purification and Biochemical Characterization of the Bacteriophage λ Holin, Texas A&M University, College Station, TX
4. Bonovich, M. T., and Young, R. (1991) J. Bacteriol. 173, 2987–2995
5. Blasi, U., Fratis, P., Chang, C.-Y., Zhang, N., and Young, R. (1999) J. Bacteriol. 181, 2922–2929
6. Raab, R., Neal, G., Garrett, J., Grimalia, R., Fusselman, R., and Young, R. (1990) J. Bacteriol. 167, 1035–1042
7. Johnson-Bonz, R., Chang, C.-Y., and Young, R. (1994) Molec. Microbiol. 13, 495–504
8. Reader, R. W., and Siminovitch, L. (1971) Virology 43, 607–622
9. Raab, R., Neal, G., Sotahsky, C., Smith, J., and Young, R. (1988) J. Mol. Biol. 199, 95–105
10. Blasi, U., Nam, K., Hartz, D., Gold, L., and Young, R. (1989) EMBO J. 8, 3501–3510
11. Blasi, U., Chang, C.-Y., Zagotta, M. T., Nam, K., and Young, R. (1990) EMBO J. 9, 981–989
12. Blasi, U., and Young, R. (1996) Mol. Microbiol. 21, 675–682
13. Chang, C.-Y., Nam, K., and Young, R. (1995) J. Bacteriol. 177, 3283–3294
14. Smith, D. L., Struck, D. K., Scholtz, J. M., and Young, R. (1998) J. Bacteriol. 180, 2531–2540
15. Graschopf, A., and Blasi, U. (1999) Mol. Microbiol. 33, 569–582
16. Barenboim, M., Chang, C.-Y., 4th Haj F., and Young, R. (1999) Mol. Microbiol.

4 M. Barenboim and R. Young, unpublished data.
Three Transmembrane Domains for λ S

27. Krishnasastry, M., Walker, B., Braha, O., and Bayley, H. (1994) FEBS Lett. 356, 66–71
28. Smith, D. L., and Young, R. (1998) J. Bacteriol. 180, 4199–4211
29. Zagotta, M. T., and Wilson, D. B. (1990) J. Bacteriol. 172, 912–921
30. Chang, C.-Y. (1994) Synthesis, Function and Regulation of the Lambda Holin, Texas A&M University, College Station, TX.
31. von Heijne, G., and Manoil, C. (1990) Protein Eng. 4, 109–112
32. Fu, D., and Maloney, P. C. (1998) J. Biol. Chem. 273, 17962–17967
33. Matos, M., Fann, M. C., Yan, R. T., and Maloney, P. C. (1996) J. Biol. Chem. 271, 18571–18575
34. Cao, G., Kuhn, A., and Dalbey, R. E. (1995) EMBO J. 14, 866–875
35. Wülfing, C., Lombardero, J., and Pluckthun, A. (1994) J. Biol. Chem. 269, 2895–2901