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Dual Start Motif in Two Lambdoid *S* Genes Unrelated to λ *S*

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The lysis gene region of phage 21 contains three overlapping reading frames, designated S^{21}, R^{21}, and Rz^{21} on the basis of the analogy with the *SRRz* gene cluster of phage λ. The 71-codon S^{21} gene complements λ *Sam7* for lysis function but shows no detectable homology with S^{λ} in the amino acid or nucleotide sequence. A highly related DNA sequence from the bacteriophage PA-2 was found by computer search of the GenBank data base. Correction of this sequence by insertion of a single base revealed another 71-codon reading frame, which is accordingly designated the S^{PA-2} gene and is 85% identical to S^{21}. There are thus two unrelated classes of *S* genes: class I, consisting of the homologous 107-codon S^{λ} and 108-codon P22 gene *13*, and class II, consisting of the 71-codon S^{21} and S^{PA-2} genes. The codon sequence Met-Lys-(X)-Met. . . begins all four genes. The two Met codons in S^{λ} and *13* have been shown to serve as translational starts for distinct polypeptide products which have opposing functions: the shorter polypeptide serves as the lethal lysis effector, whereas the longer polypeptide acts as a lysis inhibitor. To test whether this same system exists in the class II *S* genes, the Met-1 and Met-4 codons of S^{21} were altered in inducible plasmid clones and the resultant lysis profiles were monitored. Elimination of the Met-1 start results in increased toxicity, and lysis, although not complete, begins earlier, which suggests that both starts are used in the scheduling of lysis by S^{21} and is consistent with the idea that the 71- and 68-residue products act as a lysis inhibitor and a lysis effector, respectively. In addition, the *R* gene of 21 was shown to be related to P22 gene *19*, which encodes a true lysozyme activity, and was also found to be nearly identical to PA-2 *ORF2*. We infer that the 21 and PA-2 *R* genes both encode lysozymes in the T4 *e* gene family. These three genes form a second class of lambdoid *R* genes, with the λ *R* gene being the sole member of the first class. The existence of two interchangeable but unrelated classes of *S* genes and *R* genes is discussed in terms of a model of bacteriophage evolution in which the individual gene is the unit of evolution.

The lambdoid phages share general features of genetic organization and physiological behavior. Genes involved in each of the fundamental processes of the lambdoid life-style, i.e., lysogeny, recombination, DNA replication, early and late gene control, particle assembly, and lysis, are clustered together on the genome. Hybrid phages are easily constructed by recombination between these gene groups. Within a functional grouping, however, genes of identical function from two different lambdoid phages may or may not possess a detectable evolutionary relationship. The lysis gene group, consisting in phage lambda of the genes *S*, *R*, and *Rz*, is illustrative (Fig. 1).

The *S* gene controls lysis by virtue of its two opposing gene products, S105 and S107 (3, 4). S105, which is expressed from initiations at codon Met-3 of the 107-codon reading frame, is a functional lysis effector that oligomerizes to form holes in the inner membrane. S107, produced from translational initiation events at Met-1, acts to inhibit pore formation by S105. The partition of translational starts between Met-1 and Met-3 is, at least in part, controlled by an RNA stem-loop structure, designated as the *sdi* site (Fig. 1), which overlaps the ribosome-binding site for Met-1 (4). The *R* gene encodes a soluble, monomeric transglycosylase which accumulates in the cytoplasm during late gene expression. This enzyme cleaves the 1-4 glycosidic bonds in murein not by hydrolysis, as with canonical lysozyme activity, but rather by internal transfer of the reducing group of muramic acid to carbon 6 of the same molecule, yielding 1,6-anhydro-*N*-acetyl-muramic residues (1). The formation of the *S*-mediated pores triggers lysis by releasing the *R* gene product into the periplasm, thereby causing the rapid destruction of the peptidoglycan. The *Rz* function may account for the endopeptidase activity, found in λ infections and originally ascribed to *R*, which cleaves the oligopeptide cross-links in the peptidoglycan (26). It is required for lysis only in the presence of divalent cations and may be involved in the cleavage of oligopeptide cross-links between the outer membrane and the peptidoglycan (6, 27).

There are three analogous genes in the *Salmonella* phage P22: genes *13*, *19*, and *15*, each with a different evolutionary relationship to the lambda cistrons (Fig. 1). Gene *13* consists of 108 codons and is 89% identical to *S*, and the two-product motif is conserved, with starts at Met-1 and Met-4 producing a lysis inhibitor and effector, respectively (15). In addition, P22 has an RNA stem-loop structure analogous in form, function, and location to the λ *sdi* site (15). Gene *15* has 59% amino acid sequence identity with *Rz*, and lesions in *15* prevent lysis in the presence of divalent cations in *Salmonella typhimurium* (6). In contrast, gene *19* has no detectable similarity to *R* and encodes a lysozyme similar to the T4 *e* gene product, which hydrolyzes glycosidic bonds in the peptidoglycan (20). The lambda *R* and P22 *19* genes thus encode proteins with similar masses, charges, and roles (destruction of the peptidoglycan) but with different enzymatic activities and presumably different three-dimensional structures. Complementation has been shown for the *S-13* and *R-19* gene pairs and presumably would also apply for the *Rz-15* genes (15, 20). Since these genes are contiguous and, in most cases, slightly overlapping, the evolution of the "lysis cassette" in these two lambdoid phages is puzzling. In this report, the structure and function of the lysis gene region

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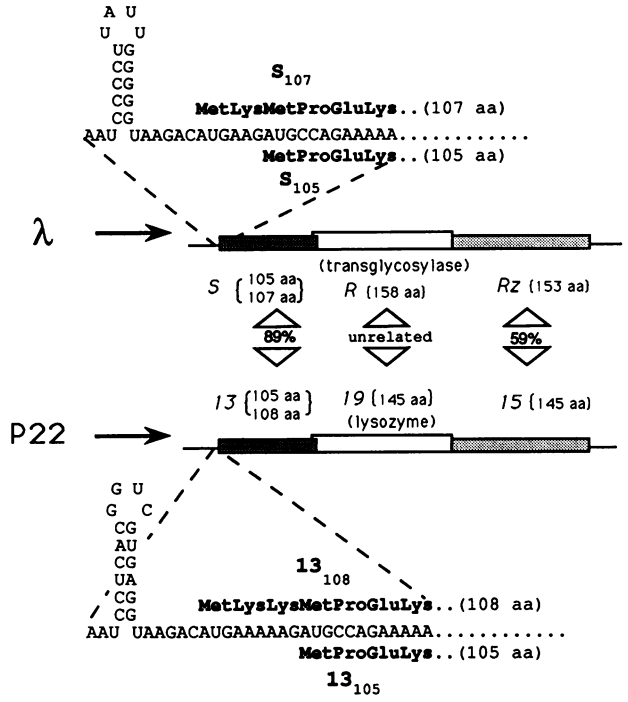

FIG. 1. Dual start motifs regulate lysis in λ and P22. The lysis gene regions of λ and P22 are shown. In both bacteriophages, the three lysis genes are the first cistrons transcribed from the late promoter (large arrows). Genes *S* and *13* both have two start codons (codons 1 and 3 in *S* and codons 1 and 4 in *13*), both of which function in vivo, resulting in two protein products in each case: S107 and S105 from the 107-codon *S* reading frame and 13_{108} and 13_{105} from the 108-codon *13* reading frame. The 5′ region of the *S* cistron of λ and the *13* cistron of P22 are enlarged to show the *sdi* stem-loop structure which controls the partition of translational initiation events at the two start codons. The next cistron downstream, *R* in λ and *19* in P22, encodes a peptidoglycan-degrading enzyme: a true lysozyme in *19* and a transglycosylase in *R*. The third cistron, *Rz* in λ and *15* in P22, has an unknown activity but is required for lysis in the presence of divalent cations. The percent identities in the protein sequences are indicated between the open triangles. In each case, there is overlap between the adjacent cistrons, ranging from 1 to 17 bp. aa, amino acids.

of another lambdoid phage, 21, is described and compared with those of lambda and P22. The comparison has implications both for the evolution of bacteriophages and for the mechanism and regulation of the lytic event.

MATERIALS AND METHODS

Bacteriophages, plasmids, and bacterial strains. The lambdoid phages 21, 82, and 434 were obtained as lysogens of the prototrophic K-12 strain W3101 from J. Roberts (Cornell University, Ithaca, N.Y.), as was the plasmid pHCG120 (Fig. 2). The plasmid pTZ18R was obtained from Pharmacia, and the phagemid Bluescript M13− was obtained from Stratagene (Fig. 2). λΔ(*SR*) has the complete genotype λ*b519 b515* Tn*903 cI857 nin5* Δ(*SR*), carrying a deletion of all of *S* and part of *R* (the deletion end points are drawn to scale in Fig. 2) (18). λCE6 is λ*int*::(T7 gene *1*) *cI857 Sam7*, in which the T7 gene *1* for RNA polymerase is inserted into the *int* gene, oriented to be expressed by transcription from the lambda p_I and p_L promoters (25). MC4100 is Δ*lac rpsL*. CQ21 has the genotype *ara leu lacI*^{q1} *purE gal his argG rpsL xyl mtl ilv* (17).

Media and culture conditions. Standard conditions for growth of cultures, plating of bacteriophage lambda, thermal induction of lysogens, and monitoring of lysis profiles have been described previously (4). All cultures used for bacteriophage lambda infections or plating were grown fresh overnight in TB (10 g of Bacto-Tryptone [Difco], 5 g of NaCl per liter). All plasmids with inducible *lacPO* control regions were propagated in CQ21. Induction with IPTG (isopropyl-β-D-thiogalactopyranoside) was done by addition of a 1 M sterile stock to the culture volume for a final concentration of 1 mM. Inducing agar plates also contained 1 mM IPTG.

Isolation of λ hybrid *QSR* recombinants. Hybrid *QSR* recombinants were isolated by plating serial dilutions of a stock of λΔ(*SR*) on a lawn lysogenic for one of the lambdoid bacteriophages 21, 82, or 434. Single plaques arising at 10^{-2} to 10^{-3} per input particle were purified on an MC4100 indicator lawn at 30°C. Lysogens of each *QSR* hybrid were isolated by streaking for colonies from the turbid centers of the purified plaques and testing for temperature sensitivity at 42°C.

Hybridization. Southern blot hybridization was done by using as a probe nick-translated plasmid pS14 (10), which contains the *S* gene sequences (nucleotides [nt] 44,982 to 45,526 from λ) cloned under *lacPO* control in the pBR322 derivative pBH20 (13). Various bacteriophage DNAs were digested with a given restriction enzyme, resolved on 0.7% agarose gels, blotted onto nitrocellulose membranes, and hybridized overnight at 45°C with the labeled probe by using standard low-stringency conditions (21).

Isolation of the phage 21 lysis gene region. The lysis gene region of phage 21 was cloned under *lacPO* control by ligating the *Eco*RI-*Cla*I fragment from pHCG120 into the expression plasmid pTZ18R cleaved with *Eco*RI and *Acc*I (Fig. 2), thus generating the plasmid pMTB100. The plasmid pMTB101, in which the R^{21} and Rz^{21} genes are inactivated by deletion, was obtained by digesting pMTB100 with *Eco*RV and *Hin*dIII, blunt-ending with Klenow polymerase in the presence of nucleoside triphosphates (22), and ligating (Fig. 2). The plasmid pMTB200, with the phage 21 lysis genes under the transcriptional control of the T7 gene *10* promoter (25), was constructed by subcloning the phage 21 DNA out of pMTB100 as an *Eco*RI-*Hin*dIII fragment into the complementary sites in the polylinker region of plasmid Bluescript M13− (Fig. 2).

DNA sequencing and site-directed mutagenesis. The DNA sequence of bacteriophage 21 DNA from the *Eco*RI site to the *Cla*I site was determined in both directions. The fragment bounded by the *Eco*RI and *Eco*RV sites was subcloned directly into M13mp18 and M13mp19 and sequenced by standard dideoxy-sequencing methodology (23). The *Eco*RI-*Cla*I fragment was sequenced by using M13mp19 for one orientation and the phagemid Bluescript M13− (Fig. 2) for the other orientation. The latter construct was designated pMTB200 and used as a T7 expression system for analysis of lysis gene function (see below). DNA sequence analysis was performed by using the programs of the Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin, Madison (8), the IntelliGenetics Suite, Release 5.35 (IntelliGenetics Inc., Mountain View, Calif. 94040), and PC/GENE (IntelliGenetics). Site-directed mutagenesis was performed by the method of Kunkel et al. (14), by using the degenerate oligonucleotide 5′-GATTTTGT CCA(G/T)TGATTTCA(T/G)AGCAACGCC-3′ to alter the Met-1 and/or Met-4 start codons of S^{21}. Because a single

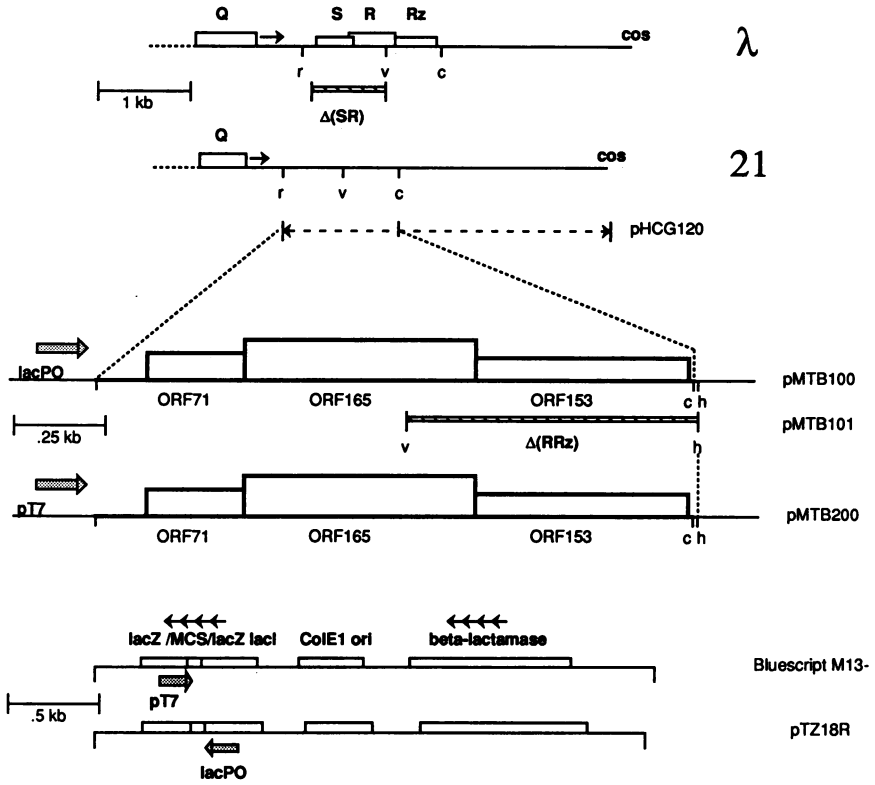

FIG. 2. Structure of lysis gene clones. The right ends of the λ and bacteriophage 21 genomes are shown. Solid arrows adjacent to the *Q* genes indicate the short, constitutive leader transcript, designated as the 6S RNA in λ and the 21a RNA in phage 21, which is antiterminated by the *Q* gene product at the onset of late gene expression. Stippled arrows indicate T7 and *lac* promoters. MCS, multiple cloning site; ori, origin of replication. Multiheaded arrows indicate the direction of expression of the *lacZ*α-MCS and β-lactamase genes on the Bluescript and pTZ vectors. Restriction sites: c, *Cla*I; *h*, *Hin*dIII; r, *Eco*RI; v, *Eco*RV. The dashed arrow indicates the length of the plasmid pHCG120, which was constructed by ligating an *Eco*RI linker to blunt-ended *cosR* of 21 genomic DNA, digesting the genomic DNA with *Eco*RI, and subcloning the 3.3-kb fragment into the unique *Eco*RI site of pBR322 (12). Hatched bars indicate deletions described in the text. Maps of the pTZ18R and Bluescript M13− expression vectors are shown linearized at the origin of the base pair numbering coordinates as defined by the suppliers (Pharmacia and Stratagene, respectively).

mutant allele, Met-1→Leu, was not obtained with this degenerate oligonucleotide in spite of repeated attempts, a second oligonucleotide 5′-GTCCATTGATTTCAGAGCAA CGCC-3′ was used to construct this mutant allele. All mutagenesis procedures were performed with the pMTB200 construct, since in this plasmid the lethal S^{21} gene is under the control of the T7 promoter, which is inactive in cells lacking T7 gene *1* (25).

Characterization of mutant S^{21} alleles. After confirmation of the alterations to the S^{21} start codons by DNA sequencing, the $(SRRz)^{21}$ region of each mutated plasmid was subcloned as an *Eco*RI-*Hin*dIII fragment (Fig. 2) into the *lacPO* expression vector pTZ18R and characterized for inducible lysis in vivo. These plasmids were designated pMTB102 (Met-1→Leu), pMTB103 (Met-4→Leu), and pMTB104 (Met-1→Leu and Met-4→Leu). Lysis profiles were also obtained directly for the mutant S^{21} alleles under T7 promoter control by a modification of the method of Studier and Moffatt (25). Exponential cultures of MC4100 carrying the pMTB200 plasmid or its mutated derivatives were grown in TB supplemented with 50 μg of ampicillin per ml, 2 mM $MgCl_2$, and 2.0 mg of maltose per ml. At A_{550} = 0.2, the cultures were infected at a multiplicity of approximately 7 with λCE6, which supplies the T7 gene *1* product. After incubation for 5 min at 37°C without shaking, the infected cultures were then aerated by vigorous shaking (culture volume was always no more than 1/10 the volume of the growth flask) and the lysis profile was monitored at A_{550}.

RESULTS

Characterization of the lysis gene region of phage 21. Both λ *S* and P22 *13* control lysis with the novel dual-product system. To determine whether the conservation of this mechanism reflected a critical role in bacteriophage function or just the 89% identity between *S* and *13*, we conducted a survey of the lysis gene structure in other lambdoid phages. To compare lytic behavior, it is convenient to use thermoinducible lysogens, so we constructed a collection of λ hybrids carrying the lysis genes from several lambdoid phages. This was accomplished by isolating plaque-forming recombinants from the plating of the defective phage λ*cI857*Δ*SR* (Fig. 2) on lawns of *Escherichia coli* lysogenic for phages 21, 80, 82, or 434. These recombinants, carrying the *cI857* thermoinducible repressor allele and the lysis gene region from each lambdoid bacteriophage, were induced in exponential phase and monitored for lysis with and without the addition of cyanide, which we have shown triggers premature lysis by subverting the inhibition function of the dual-product *S* system (3). All the recombinants showed lysis profiles very

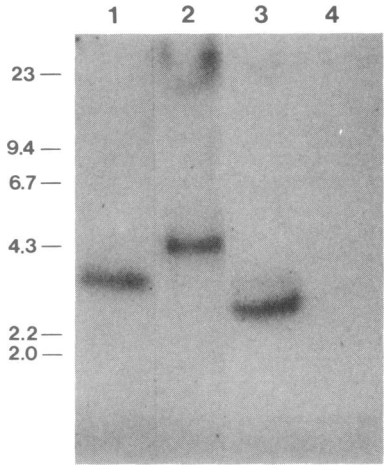

FIG. 3. Bacteriophage 21 contains no DNA hybridizable to λ *S*. DNAs purified from bacteriophages λ (lane 1), 82 (lane 2), 434 (lane 3), and 21 (lane 4) were digested to completion with *Eco*RI (lanes 1, 3, and 4) or *Hin*dIII (lane 2), resolved on 0.7% agarose gels, and subjected to Southern blot hybridization by using labeled pS14 plasmid DNA, which has the λ *S* gene cloned under the control of the *lac* promoter, as a probe. All lanes contained approximately 0.5 μg of DNA, and, although they were from different gels, they were exposed to probes of approximately the same concentrations and specific activities. The upper band in lane 2 is left-arm DNA linked by *cosL-cosR* annealing with the right-end fragment carrying the lysis gene region.

similar to λ*cI857*, and all were triggered by cyanide, suggesting that the *S*-like lysis control system was conserved in all of these bacteriophages (data not shown). Surprisingly, however, one of the hybrids, carrying the lysis genes from bacteriophage 21, showed no homology detectable by Southern blot hybridization with the *S* gene as the probe, even at reduced stringency (Fig. 3). The restriction patterns obtained from analysis of this recombinant (data not shown) were consistent with the known restriction map for the region of phage 21 DNA carrying the 21 *Q* gene and the first part of the 21 late operon (Fig. 2). The lack of hybridization was even more puzzling, since a set of restriction sites for *Eco*RI, *Eco*RV, and *Cla*I are found downstream of the Q^{21} gene in about the same spatial relationships as those which mark the lysis gene region downstream of *Q* in λ (Fig. 1). In λ, the *Eco*RI-*Cla*I fragment carries the entire *SRRz* gene cluster, and expression of this fragment from any controllable promoter is necessary and sufficient for lysis (18). The *Eco*RI-*Cla*I fragment from 21 was subcloned under the control of *lacPO* in the vector pTZ18R (Fig. 2). Cells carrying this recombinant plasmid, pMTB100, were killed when plated on inducing agar (efficiency of plating 10^{-4}) and underwent sharply defined lysis after IPTG addition to liquid culture (Fig. 4), similar to that obtained by induction of the plasmid pRG1, carrying the λ *SRRz* genes under *lacPO* control (17). Addition of 10 mM Mg^{2+} had no effect on this lysis profile (data not shown), indicating that not only were the *S* and *R* equivalents present on the fragment but that also the *Rz* equivalent was intact (6, 27). It was concluded that the *Eco*RI-*Cla*I fragment adjacent to the 21 *Q* gene carried the entire lysis gene region of phage 21.

Sequence of the phage 21 lysis genes. The sequence of the 21 lysis gene region was determined by dideoxy-nucleotide sequencing. Three overlapping reading frames were found (Fig. 2 and 5): ORF71 (nt 99 to 314), ORF165 (nt 314 to 811), and ORF153 (nt 808 to 1,269). On the basis of the analogy to λ and P22, we would expect these three reading frames to correspond to *S-13*, *R-19*, and *Rz-15*. Accordingly, ORF153 is 97% identical at both the nucleotide and amino acid

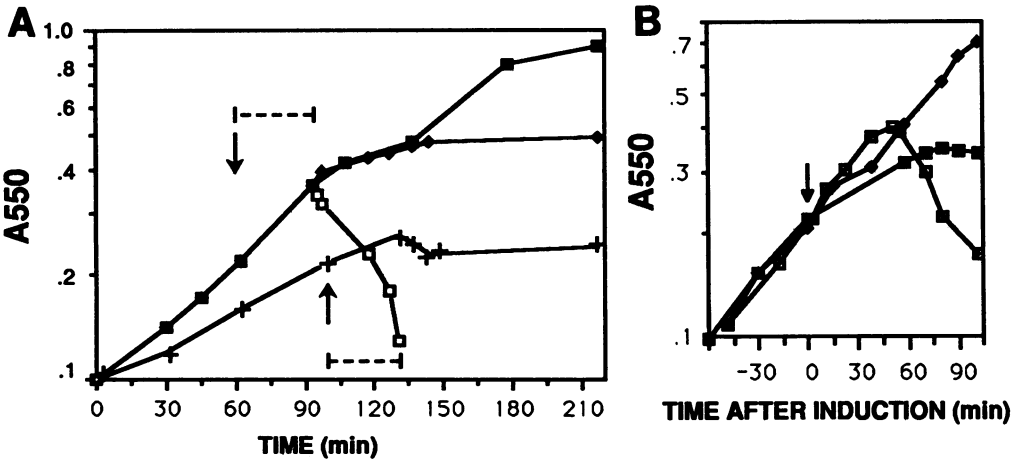

FIG. 4. (A) Induction of wild-type and mutant 21 lysis genes. Cells carrying the plasmid pMTB100, with the 21 lysis genes under *lacPO* control, were grown aerobically in minimal glycerol medium and induced at an A_{550} = 0.22 by the addition of IPTG to a final concentration of 1 mM (vertical arrows indicate time of induction. The plasmid carrying original (wild-type) *SRRz* gene cluster of phage 21 supported lysis (open squares), which begins after a 36-min lag (upper dashed bar). Isogenic plasmids carrying mutations eliminating the first start codon (pMTB102: Met-1→Leu [crosses]), the second start codon (pMTB103: Met-4→Leu [solid diamonds]), or both start codons (pMTB104: Met-1→Leu and Met-4→Leu [solid squares]) of the S^{21} gene were grown and induced in an identical manner. The lower dashed bar delimits the interval between induction of the pMTB102 culture and the onset of lysis (31 min). Times were arbitrarily measured from the point at which each culture reached A_{550} = 0.1 in logarithmic growth. (B) Complementation of λ *Sam7* by S^{21}. Cultures were grown aerobically in LB-Amp medium at 30°C, doubly induced by the simultaneous addition of IPTG to a final concentration of 1 mM and shifting to 42°C. After 20 min of shaking at this temperature, each culture was transferred to a 37°C shaker and monitored at A_{550} during subsequent aerobic incubation. Strains included CQ21(λ*cI857 Sam7*)pTZ18R (solid diamonds), CQ21pMTB101$S^{21}\Delta(R^{21}Rz^{21})$ (nonlysogenic; solid squares), and CQ21(λ*cI857 Sam7*)pMTB101$S^{21}\Delta(R^{21}Rz^{21})$ (open squares). The vertical arrow indicates the time of induction.

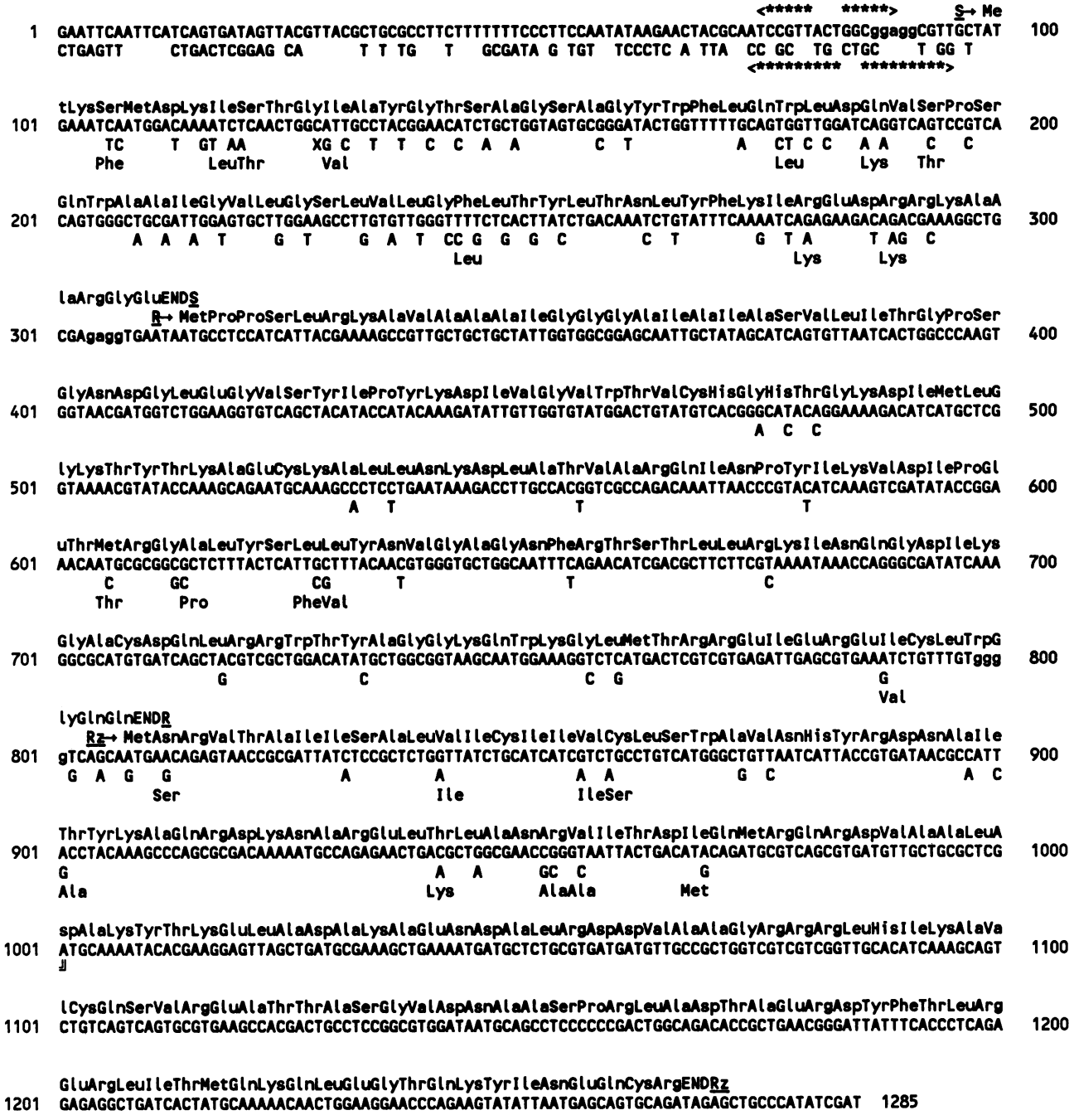

FIG. 5. Sequence of the 21 lysis gene region. The DNA sequence of the 1,285-bp *Eco*RI-*Cla*I fragment subcloned in pMTB100 (Fig. 2) is shown as a single strand running 5′ to 3′ from the *Eco*RI to the *Cla*I site. The three reading frames (Fig. 2) identified are labeled as *S* (nt 99 to 314 [ORF71]), *R* (nt 314 to 811 [ORF165]), and *Rz* (nt 808 to 1,269 [ORF153]). Below the nucleotide sequence are the DNA and predicted amino acid sequence differences, with the analogous region of bacteriophage PA-2 (2), extending from nt 1 to 1,001, where the end of the available PA-2 sequence is indicated (⇓). An X below nt 128 indicates where a base must be inserted to correct the putative error in the PA-2 sequence and allow formation of the S^{PA-2} reading frame. Lowercase letters indicate putative Shine-Dalgarno sequences for translational initiation of each cistron. The asterisk arrows indicate possible stem-loop structures which may be analogous to the *sdi* structure controlling the translational start partition of λ *S* and P22 *13* (Fig. 1).

sequence levels to the *Rz* gene of phage lambda. ORF153 was thus designated Rz^{21}. ORF165 showed 34% amino acid identity with P22 *19* (Fig. 6), indicating that 21 uses a true lysozyme for its murein degradation activity, like P22 and T4 but unlike the λ *R* transglycosylase (1, 20). Moreover, the residues conserved in the predicted amino acid sequences of ORF165 and P22 gene *19* include the glutamate common to all members of the T4 lysozyme gene family and thought to be the catalytic center of the T4 lysozyme family (Fig. 6) (16). ORF165 was designated R^{21}, to indicate that this gene

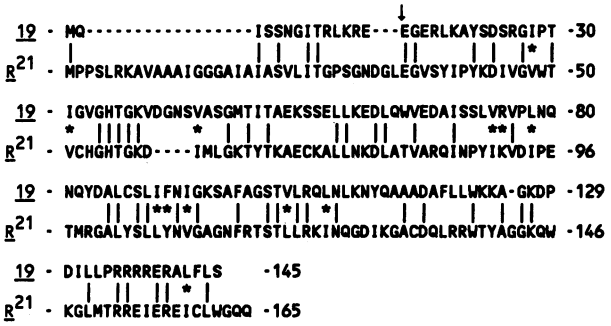

FIG. 6. Homology of the P22 lysozyme and the predicted R^{21} gene product. The predicted sequence of the R^{21} product was aligned with the sequence of P22 lysozyme (gene *19* product) by using the PALIGN program of PC/GENE, with an open gap cost of 7 and a unit gap cost of 2. The identity between *19* and R^{21} is 33.8%, and each identity is indicated by the solid line connector. Asterisks denote conservative substitutions. Gaps are indicated by dashes. The vertical arrow indicates the glutamate residue conserved in all members of the T4 lysozyme family and thought to be critical to catalysis (16).

likely served the same function in lysis as the λ *R* gene, albeit with a heterologous enzyme activity. ORF71 had no detectable sequence homology with *S*, as expected from the failure to hybridize at low stringency. However, given the lytic competency of the *Eco*RI-*Cla*I fragment and the conserved gene order of the *R* and *Rz* gene equivalents, it was likely that ORF71 was in fact S^{21}, and it was so designated.

Bacteriophage PA-2 homology. A search of the GenBank data base revealed that a region of the bacteriophage PA-2 genome showed strong sequence similarity with the 21 lysis gene region (Fig. 5). PA-2 is a temperate lambdoid bacteriophage, isolated from a porcine strain of *E. coli*, which has a different immunity, host range, and chromosomal integration site from those of λ (2). The PA-2 sequence contained a reading frame previously noted as being nearly identical with λ *Rz* and thus also very homologous with Rz^{21} (Fig. 5). Upstream of this presumptive Rz^{PA-2} is a reading frame, designated ORF2 by Blasband et al. (2), which shows 97% identity with R^{21} (Fig. 5). Thus, by extension we designate this gene as R^{PA-2}. Moreover, correcting the sequence upstream of R^{PA-2} Rz^{PA-2} by insertion of a single base pair (at nt 128) yields a 71-codon reading frame with 85% identity to S^{21}. Since the PA-2 sequence was obtained from a lysis-competent isolate of PA-2, it was likely that the original sequence reported was in error at this single nucleotide position (24). Thus, this corrected reading frame was designated S^{PA-2}.

Characterization of the S^{21} gene. To test whether S^{21} was functionally equivalent to *S*, a subclone was created in which the two downstream cistrons, R^{21} and Rz^{21}, were deleted (Fig. 2). Induction of this clone resulted in a cessation of culture growth and massive inviability but no lysis (Fig. 4B), exactly as found for analogous clones of the lambda *S* gene in the absence of functional *R* transglycosylase (11). Moreover, induction of this clone complemented the lysis defect of an induced lambda S^- lysogen (Fig. 4B). Taken with the fact that energy poisons can trigger premature lysis during the 21 vegetative period, as shown for λ *S*, we conclude that S^{21} and, by extension, S^{PA-2} indeed fulfill the same functional role in lysis as *S*.

Dual start motif regulates *S* function in phage 21. Although showing no significant sequence similarity to *S* overall, the

TABLE 1. Lysis times for bacteriophage 21 S^+ and *S*M1L alleles expressed from the T7 promoter[a]

Trial	Time of lysis onset (min postinfection)	
	pMTB201 ($S_{M1L}RRz$)	pMTB200 (*SRRz*)
1	25	42
2	35	42
3	30	41
4	38	43

[a] Cultures carrying plasmids pMTB200 or pMTB201, which have the entire 21 lysis gene region cloned downstream of the T7 promoter in the Bluescript M13− vector and are isogenic except for the alteration of the Met-1 codon of S^{21} in pMTB201, were grown. The cultures were infected with the phage λCE6, which is *Sam* and supplies the T7 gene *1* product, and monitored for the onset of lysis as described in Materials and Methods. Although the rate and extent of lysis were variable, the onset of lysis was readily detectable as the first decrease in A_{550} after infection.

S^{21} gene begins with codons specifying the sequence Met-1–Lys-2–Ser-3–Met-4. . . ., which is strikingly similar to the dual start motif defined in λ *S* and P22 *13* (Fig. 1). Moreover, the putative Shine-Dalgarno sequence for Met-1 can be drawn imbedded in an RNA secondary structure (Fig. 5), similar to the *sdi* locus demonstrated to participate in the translational regulation of *S* and *13* (4, 15). To test whether codons 1 and 4 of S^{21} serve in functions analogous to the roles of codons 1 and 3 of *S* and codons 1 and 4 of *13*, site-directed mutagenesis was performed to create a set of mutant alleles in which one or both of these start codons were eliminated. The resultant alleles were designated S^{21}M1L (Met-1→Leu), S^{21}M4L (Met-4→Leu), and S^{21}M1/4L (Met-1, Met-4→Leu). Cultures of cells carrying plasmids with the wild-type and mutant S^{21} alleles under *lacPO* control were induced, and the lysis profiles were monitored (Fig. 4A). The S^{21}M4L and S^{21}M1/4L alleles did not support lysis, whereas both the S^{21+} and S^{21}M1L alleles were lysis competent (Fig. 4A), consistent with the notion that the Met-4 start codon is the initiation site for the S^{21} lysis effector protein, which would be designated as $S^{21}68$ to be consistent with the terminology established for the lambda S105 lysis effector (18). Under these conditions, induction of the S^{21}M1L allele did result in lysis earlier than that of the wild type, but the uninduced culture grew at a reduced rate and, upon induction, showed only partial lysis (Fig. 4A). This physiological behavior is typical of cells in which the lambda *S* gene is insufficiently repressed on a multicopy plasmid, and the partial lysis results from a high proportion of cured cells in the culture (18, 19). These data indicate that the S^{21}M1L allele is more toxic than the other S^{21} alleles, assuming the basal level of transcription is the same in these isogenic plasmids. This result supports the idea that Met-1 is for S^{21}, as for lambda *S*, the start codon for a lysis inhibitor, which would be $S^{21}71$ (the analog of lambda S107).

By using the plasmid pMTB200 and derivatives, in which the 21 *SRRz* genes were under the control of the T7 promoter (Fig. 2), basal level expression of these genes could be reduced to negligible levels. Under these conditions, with T7 RNA polymerase supplied by infection, the lysis profiles of these four alleles could be compared without the complication of preinduction toxicity. Because of the necessity for preabsorption of the λCE6 gene *1* vector, the infection protocol is inherently more variable than the induction protocols and gave less complete lytic profiles (data not shown). Nevertheless, Table 1 shows that S^{21}M1L reproduc-

ibly supports a more rapid lysis than wild-type S^{21} by using this experimental system.

DISCUSSION

We have described the lysis gene region of phage 21, consisting of the homologs of the lambda *SRRz*. This has led to the positive identification of the very similar PA-2 *R* gene, previously reported as an open reading frame (ORF) of unknown function (2). In addition, the S^{21} gene, although of no detectable sequence similarity with lambda *S*, has been shown to complement *Sam* in induced lambda lysogens. The identification of S^{21} has led to the identification of a highly similar *S* gene in PA-2, after minor correction of the previously reported DNA sequence. Finally, inductions of mutant S^{21} alleles constructed by site-directed mutagenesis suggest that the S^{21} Met-1 and Met-4 codons may serve the same function as the two start codons in lambda *S*. This conclusion must be regarded as tentative, however, in view of the partial lysis supported by the S^{21} allele in which the Met-1 start codon was eliminated. It is likely that this ambiguous result is due to the increased basal toxicity of the M1L allele, but final confirmation must await a direct demonstration that the M4L allele in *trans* can inhibit S^{21}-mediated lysis, as previously done for the M3L allele of S^{21} (3). In any case, the data are at least consistent with the notion that S^{21} produces two gene products, $S^{21}71$ and $S^{21}68$, with the shorter gene product functioning as the lethal lysis effector and the longer product serving as a lysis inhibitor. These findings have implications for the mechanism of *S*-mediated lysis and for the evolution of bacteriophages.

S^{21} and *S*: unrelated lysis control genes with dual start regulation. The dual start regulatory motif recently established for the lambda lysis control gene *S* and conserved in the closely related gene *13* of P22 has now been found in the functional homolog of *S* in phage 21, although there is no detectable relationship between the DNA and predicted amino acid sequences of the two cistrons. This may represent convergent evolution to the dual start motif in the two types of *S* genes or extremely strong conservation of the dual start motif during total divergence of the rest of the sequences. In either case, it seems clear that the motif confers a strong selective advantage, a conclusion which is not obviously consistent with the fact that mutational loss of the Met-1 codon has no significant effect on plating efficiency or plaque size in phage lambda (18). However, it has been shown that plaque size is an insensitive indicator of *S* gene function, since within certain limits, from 10 min sooner than the normal 40-min lysis time to more than 60 min later, plaque size is unaffected, presumably because changes in burst size compensate for the reduced or increased number of infective cycles during the incubation of the plaque assay plate (18). Nonetheless, it seems incongruous that such a powerful evolutionary selection could be associated with the dual start motif, given the nearly cryptic vegetative phenotype. Recently, we suggested that the major role of the dual start regulatory system is not in the vegetative but rather in the prophage state, either during lysogenic growth or in its establishment or both (15). In lambdoid phages, the *S* lysis control gene lies immediately downstream from the very strong constitutive late promoter, p_R, in lambda, which is under the antitermination control of the *Q* gene product (Fig. 2). According to this rationale, the dual start motif serves either to protect the lysogenic cell from occasional readthrough of the late terminator or to prevent toxic expression of *S* from *N*-mediated transcription during early protein synthesis, before the lysis-lysogeny decision has been made or implemented. The evident toxicity of S^{21}M1L under *lacPO* control (Fig. 4A) supports this idea, as does the poor maintainability of lysogens of lambda phages carrying the *S*M1L allele (19). Experiments are under way to test this notion.

Model for S protein disposition in the inner membrane. The S^{21} gene has only 71 codons, with two regions in the predicted amino acid sequence which consist of at least 20 hydrophobic residues, suggesting that there are two membrane-spanning helical domains (Fig. 7A). In the past, we have drawn models for λ S disposition in the membrane in two different ways: first, with three membrane-spanning domains (18), taking the charge-free region between residues 64 and 84 as imbedded in the membrane, and, more recently, with two membrane-spanning domains, which allows the insertion of the S protein as a pair of helices, a commonly utilized mode of membrane imbedding (9). Although there is still no biochemical data, it seems likely that the two-domain model is correct, assuming *S* and S^{21} are disposed in an analogous manner. Drawing the models this way also makes it possible to compare the two dissimilar amino acid sequences and make reasonable deductions as to which features are required for S function. Both *S* and S^{21} would have no net charge at the N terminus, other than the positively charged Lys-2 at the N terminus of the S107 and $S^{21}71$ inhibitor molecules. This is consistent with the idea that it is the presence of the net positive charge at the N terminus which determines the inhibitory properties of the longer gene product (3). Beginning from the N terminus, both molecules would have no net charge in the N-proximal membrane-spanning helix, and both would have hydrophilic residues near the middle of this domain (the Lys-17–Glu-18 ion pair in *S* and Ser-16 in S^{21}). Both would have hydrophilic turn regions projecting at the periplasmic interface, and both would have net-neutral-charge membrane-spanning domains returning to the cytoplasm. Finally, both would have highly hydrophilic, positively charged C-terminal domains: in *S*, 7 of 16 residues are charged, with a net charge of +4, in S^{21}, 9 of 13 residues are charged, with a net charge of +2. Why S has the extra domain (approximately residues 66 to 85) is unknown. Most of this region has been refractory to mutational analysis (17), suggesting that this domain does not play a crucial role in S function.

Implications for the evolution of lambdoid bacteriophages. On the basis of the near identity of lambda *S* and P22 *13* and the unrelatedness of lambda *R* and P22 *19*, Botstein (5) concluded that the unit of evolution in phages was the gene, rather than a functional grouping of genes. There are now four lambdoid lysis gene regions for which extensive sequence information is available: lambda (7), P22 (6, 20), 21, and PA-2 (2). Two different, evolutionarily distinct classes of *S* genes (lambda *S* and P22 *13* in class I and the *S* genes of 21 and PA-2 in class II), two different classes of *R* genes (lambda *R* in class I and the P22, 21, and PA-2 *R* genes in class II), and a widely conserved *Rz* gene have been found to constitute the lysis cassettes of these phages. By using these class definitions, the lysis genotypes of lambda, P22, 21, and PA-2 can be written as I-I-Z, I-II-Z, II-II-Z, and II-II-Z, respectively. The existence of these combinations supports the Botstein hypothesis. One would predict that a lysis cassette with the II-I-Z genotype could be found, since the S^{21}-*R* combination has already been demonstrated to be lytically functional (Fig. 4), and it would be the reciprocal recombinant in the evolutionary event which produced the I-II-Z array found in P22. It should be pointed out, however,

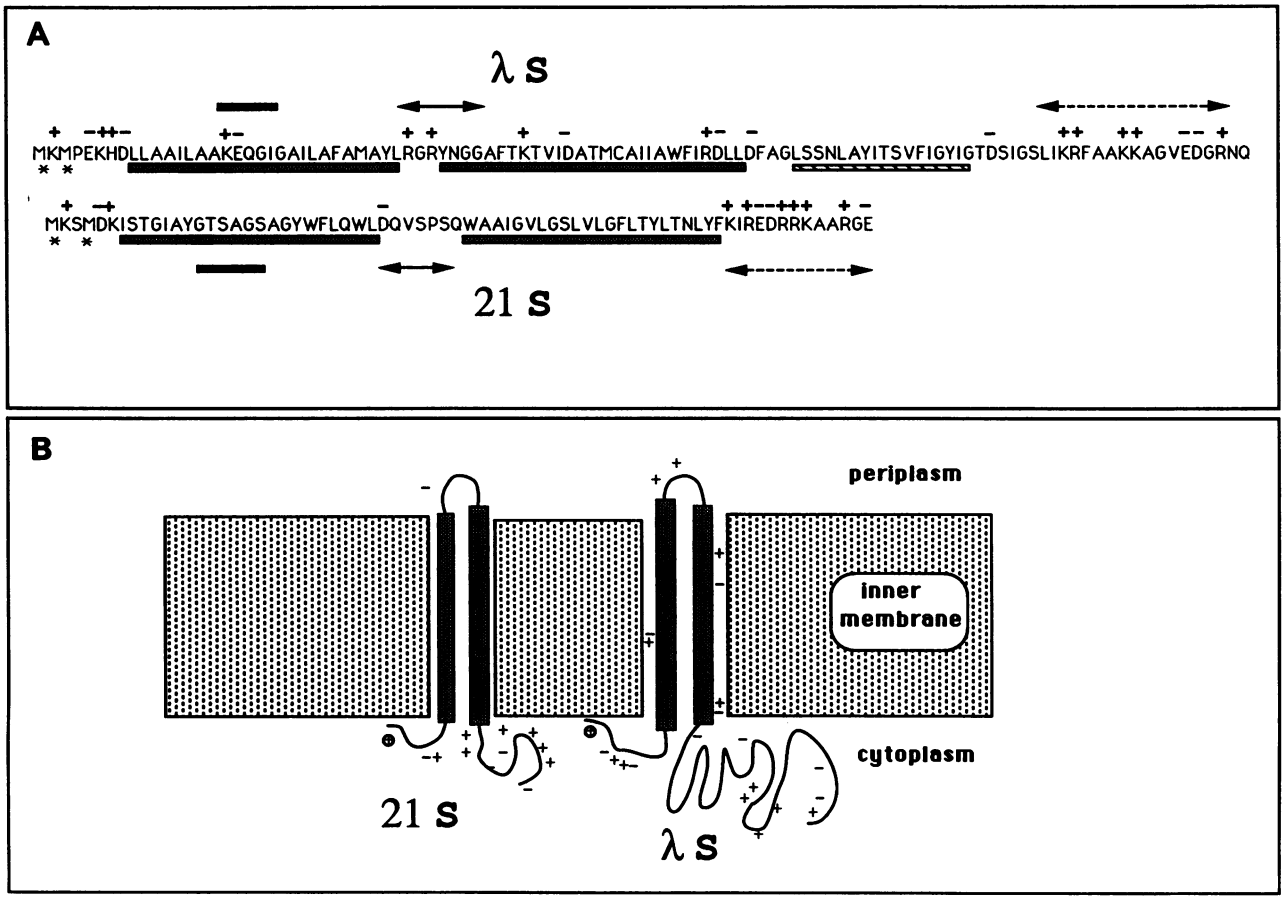

FIG. 7. Comparison of primary sequences of predicted *S* proteins from λ and 21. (A) *S* and S^{21} protein sequences are displayed in single-letter code, with charged residues indicated by + and −. Stippled bars indicate putative membrane-spanning domains of at least 20 residues with no net charge. The thin hatched bar under the *S* sequence indicates a third region formerly proposed as a membrane-spanning domain in a model for *S* disposition in the inner membrane (17). Dashed arrows indicate highly charged regions found in both protein sequences. Solid arrows denote the putative turn regions separating the two putative membrane-spanning domains. The solid bars cover regions which are rich in glycine and hydrophilic residues and internal to the amino-proximal membrane-spanning domains. Asterisks indicate the two Met residues in each protein which are N terminal in the long and short forms of the gene products. (B) Model for the disposition of the λ and 21 *S* proteins (the inhibitor form of each is shown; i.e., S107 and $S^{21}71$, respectively) in the inner membrane.

that these gene exchanges within the lysis gene grouping imply some kind of recombination sequence at the *S-R* and *R-Rz* junctions which are not obvious from inspection of the sequence. Indeed, the fact that the lysis gene cluster in each lambdoid phage is an "overlappon," consisting of reading frames which overlap from 1 to 17 base pairs, puts severe constraints on the potential recombinational junctions. Thus, the mode by which these functionally interdependent genes reassort remains a mystery.

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