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The lethal λ *S* gene encodes its own inhibitor

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The 107 codon reading frame of the λ lysis gene *S* begins with the codon sequence Met1–Lys2–Met3 . . . , and it has been demonstrated *in vitro* that both Met codons are used for translational starts. Furthermore, the partition of initiation events at the two start codons strongly affects the scheduling of lysis. We have presented a model in which the longer product, S107, acts as an inhibitor of the shorter product, S105, the lethal lysis effector, despite the fact that the two molecules differ only in the Met–Lys residues at the amino terminus of S107. Using immunological and biochemical methods, we show in this report that the two predicted protein products, S105 and S107, are detectable *in vivo* as stable, membrane-bound molecules. We show that S107 acts as an inhibitor in *trans*, and that its inhibitory function is entirely defined by the positively charged Lys2 residue. Moreover, our data show that energy poisons abolish the inhibitory function of S107 and simultaneously convert S107 into a lysis effector. We propose a two step model for the lethal action of gene *S*: first, induction of the *S* gene results in the accumulation of S105 and S107 molecules in mixed oligomeric patches in the cytoplasmic membrane; second, S monomers rearrange by lateral diffusion within the patch to form an aqueous pore. The *R* gene product, a transglycosylase, is released through the pore to the periplasm, resulting in destruction of the peptidoglycan and bursting of the cell. According to this model, the lateral diffusion step is inhibited by the energized state of the membrane. A simple basis for this inhibition could be the ionic interaction between the negatively charged inner surface of the cytoplasmic membrane and the positively charged Lys2 residue of S107. Dissipation of the membrane potential by formation of a functional pore abolishes this interaction and allows rearrangement of S105/S107 hetero-oligomers. Thus, not only do the two protein products of the *S* gene have opposing function but S107 itself has a dual capacity. S107 acts as a lysis inhibitor as long as the infected cell can maintain the membrane potential, and it also contributes actively to pore formation when the membrane potential collapses, i.e. once a single lysis pore has been formed. Hence, the function of the *S* gene is inherently saltatory, so that progeny phage are efficiently released in the lytic burst.

Key words: lysis/inner membrane/pore formation

Introduction

Lysis of cells infected by phage λ requires the function of the *S* (107 codons) and *R* (155 codons) genes, which are the first two genes on the late transcript (Harris *et al.*, 1967; Reader and Siminovitch, 1971a). The 17 kd *R* gene product is a transglycosylase, or 'endolysin' (Bienkowska-Szewczyk *et al.*, 1981), which accumulates in the cytoplasm from the beginning of the late transcription of 8–10 min after infection. After ~30 min of accumulation (or 40 min after infection), gpR is suddenly released to the periplasm. Since endolysin is synthesized in great excess, this release to the periplasm causes a rapid disruption of the peptidoglycan and bursting of the cell. The *S* gene product is required for this sudden release of the *R* transglycosylase. If the *S* gene is defective, transglycosylase activity continues to accumulate in the cytoplasm beyond 40 min and lysis is not observed. Moreover, macromolecular synthesis in the infected cell continues unabated. In the absence of *R* function, respiration and macromolecular synthesis cease abruptly at 40 min, but lysis does not occur. These observations and other lines of evidence suggest that the *S* gene product oligomerizes and forms a hydrophilic pore in the inner membrane (Reader and Siminovitch, 1971b; Garrett *et al.*, 1982; Wilson, 1982; Zagotta and Wilson, 1990). The structure of the pore, however, is not known and its sudden formation after 30 min of *S* gene transcription is not understood.

To gain insight into the structure of the pore and how its formation is regulated, the *S* gene was subjected to a mutational analysis (Raab *et al.*, 1986). Nearly one-third of missense alleles selected for reduced lethality showed dominant character, consistent with the proposed oligomeric structure of the *S* pore (Raab *et al.*, 1988). Surprisingly, several alleles with dominant character were found to have single base changes just upstream of the reading frame. These mutations were shown to be located in a stem–loop secondary structure, designated *sdi* (*s*tructure *d*irected *i*nitiation), which was implicated in the translational regulation of *S* (Raab *et al.*, 1988). Further analysis utilizing site-directed mutagenesis and primer extension inhibition, or 'toeprinting', has led us to propose a detailed molecular model for the translational control of gene *S* (Bläsi *et al.*, 1989). In this model, the 5′ stem–loop, an internal stem–loop and two adjacent Shine–Dalgarno sequences, which we now collectively redesignate as the *sdi* locus (Figure 1), participate together in the partition of translation initiation events between two start codons, Met1 and Met3. Initiation at Met1 results in the synthesis of a 107 residue polypeptide, S107. Initiation at Met3 results in a 105 residue polypeptide, S105, lacking the N-terminal Met1–Lys2 residues of S107. The timing of lysis *in vivo* was shown to be a function of the partition of translational initiation events between Met1 and Met3, as determined by *in vitro* toeprinting. Hence, S105 and S107 were proposed to have opposing function: S105 acting as the lethal lysis effector and S107 acting as the lysis antagonist.

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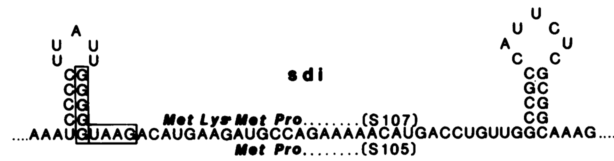

Fig. 1. Translational start region of the λ *S* gene. The boxed sequences GGGGG and UAAG, complementary to the 3′ end of the 16S rRNA, serve as Shine–Dalgarno sequences for initiations at Met1 and Met3, respectively (Bläsi *et al.*, 1989). The amino-terminal amino acid sequences of the two *S* gene products are given above (S107) and below (S105) the mRNA sequence. The entire initiation region containing the two adjacent Shine–Dalgarno sites, the two stem–loop structures, upstream and downstream of the start codons, and the intervening 37 nucleotides of single-stranded mRNA is designated as *sdi* locus.

Here we use an *in vitro* translation system, immunological methods and a T7 expression system to provide direct molecular evidence for the synthesis and the subcellular localization of the two *S* gene products. Furthermore, site-directed mutagenesis was employed to define the basis for the inhibitory properties of the amino terminus of S107. A model for the inhibitory mode of action of the S107 molecule is presented.

Results

In vitro translation of S^+, S105 and S107 alleles

It has been estimated that at the time of lysis only ~1000 molecules of protein S are present in the cell (Zagotta and Wilson, 1990; R.Raab and R.Young, unpublished data). To facilitate the identification of both *S* gene products, transcripts derived from a set of plasmids (Tables III and IV), pBS108 (*S105*), pBS109 (*S107*), pBS110 (S^+) and pBS122 (S^-) were translated *in vitro*. The [^{35}S]methionine labeled proteins were visualized by autoradiography after separation on a 17.5% SDS–polyacrylamide gel as described in Materials and methods. The electrophoretic conditions employed allowed the separation of the S105 and the S107 products (Figure 2, lane 4), although differing only by two amino acid residues. Translation of the pBS109 transcript (carrying the Met3 → Leu change) and the pBS108 transcript (carrying the Met1 → Leu change) revealed only signals for proteins S107 and S105, respectively (Figure 2, lanes 2 and 3). No S-specific bands were visible after expression of the Met1,3 → Leu allele (pBS122, in which both start codons have been eliminated; Figure 2, lane 1).

Both forms of protein S are membrane bound

S protein, not resolved as S107 and S105, has been detected in the inner membrane of *Escherichia coli* (Altman *et al.*, 1983, 1985). To visualize both *S* gene products *in vivo* and to determine the subcellular localization of the minor *S* species, S107, two different approaches were taken.

First, membrane samples were prepared 65 min after induction of λ S^+ or λ S^- lysogens and the proteins were separated by their charge properties using non-equilibrated pH gradient gel electrophoresis (NEPHGE). The predicted isoelectric points for S105 and S107 are 9.22 and 9.60, respectively. The Western blot of the NEPHGE gels probed with anti-*S* antibodies is shown in Figure 3A. Two signals were visible in the membrane extracts obtained from λ S^+ lysogens (Figure 3A, lane 1); no S-specific bands were

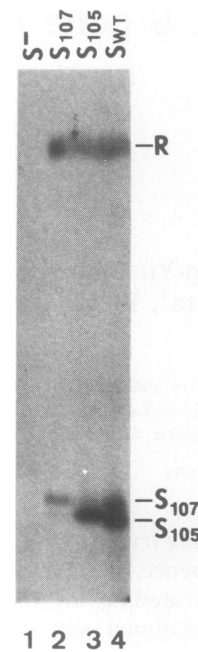

Fig. 2. Resolution of proteins S105 and S107. The *in vitro* translation products of transcripts carrying the S^- (Met1,3 → Leu) allele, the *S107* (Met3 → Leu) allele, the *S105* (Met1 → Leu) allele or gene S^+ along with the *R* and *Rz* genes are shown in **lanes 1, 2, 3** and **4**, respectively. The positions of the *S* gene products, S105 and S107, and of protein R are indicated at the right.

observed in the negative control (Figure 3A, lane 2). As predicted from our recent data, in which initiations at Met3 are favored over Met1 (Bläsi *et al.*, 1989), the more abundant species is the more acidic form, S105.

Second, an *in vivo* T7 expression system was employed to enhance the production of S105 and S107 in order to separate both forms by molecular mass. Three different *S* alleles S^+, *S107* and S^- encoded by plasmids pBS110, pBS109 and pBS122 (Tables III and IV), respectively, were used in this study. Since the three *S* alleles are cloned under control of a T7 promoter, they are expressed after infection with the λ phage CE6, which carries T7 gene *1* (RNA polymerase). The cells were labeled and processed as described in Materials and methods. Both S products were found in the membrane fraction (Figure 3B, lanes 4 and 6). No S protein was detected in the cytoplasmic fraction (Figure 3B, lanes 3 and 5). It should be noted that the utilization of plasmid pBS108, which encodes only S105, did not allow visualization of this product. Cells carrying plasmid pBS108 lysed immediately after infection with λ CE6, most likely due to the severe toxicity of the S105 protein.

The proteins S105 and S107 are stable

The possibility existed that protein S107 is post-translationally processed, i.e. converted to the lethal lysis effector S105. To demonstrate the stability of both S products a pulse–chase experiment was carried out. *E.coli* MC4100 carrying plasmid pBS125 (Table II), which, when compared to the S^+ plasmid pBS110, produces the same amount of S105 but a significantly higher amount of S107 (Bläsi *et al.*, 1989), was infected with λ CE6. The pulse labeling was followed by a 10–80 min chase in the presence of chloramphenicol and unlabeled methionine. Figure 4 shows

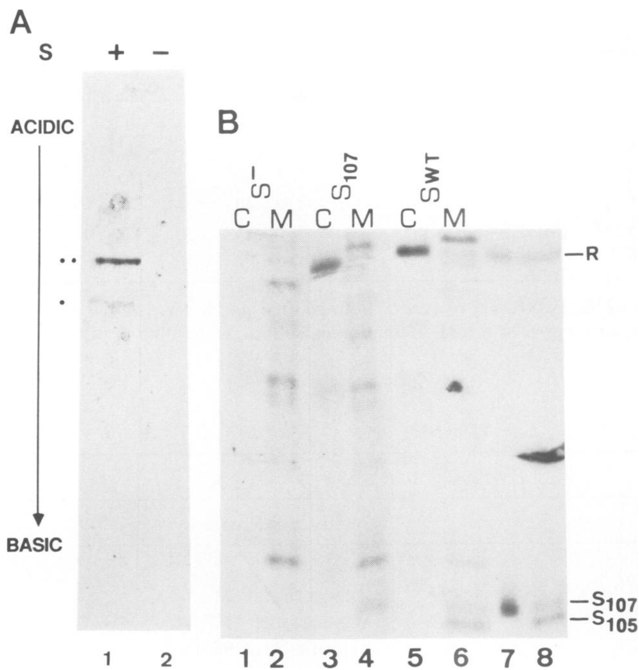

Fig. 3. Membrane localization of proteins S105 and S107. (**A**) Western blot analysis of the NEPHGE gel separating the S105 and S107 polypeptides by charge. Membrane samples derived from cultures of induced λ S^+R^- (lane 1) and λ S^-R^- (lane 2) lysogens were prepared 65 min after induction and run on a NEPHGE gel as described in Materials and methods. The immunoblot was incubated with anti-S antibodies and the S-specific bands were visualized by standard procedures with anti-rabbit IgG conjugated with alkaline phosphatase. The two dots mark the position of the more acidic S105 protein (pI 9.22). The position of the S107 protein (pI 9.60) is marked by a single dot. (**B**) Labeling of the S105 and S107 polypeptides *in vivo*. *E. coli* strain MC4100 harboring plasmids pBS109 (*S107*), pBS110 (S^+) or pBS122 (S^-) was grown in minimal media, infected with λ CE6 (carries T7 gene *1*) and labeled with [^{35}S]methionine as described in Materials and methods. Then the cytoplasmic fraction (C) and the membrane fraction (M) were isolated and the samples were analyzed on a 17.5% SDS–polyacrylamide gel. The proteins S105 and S107 were only detected in the membrane fraction (lanes 4 and 6). No S protein was detected in the cytoplasmic fraction (lanes 3 and 5) or in the S-negative control (lanes 1 and 2). Lanes 7 and 8, *in vitro* translated *S107* and S^+, respectively. The positions of the two S polypeptides, S105 and S107, and of protein R are given at the right.

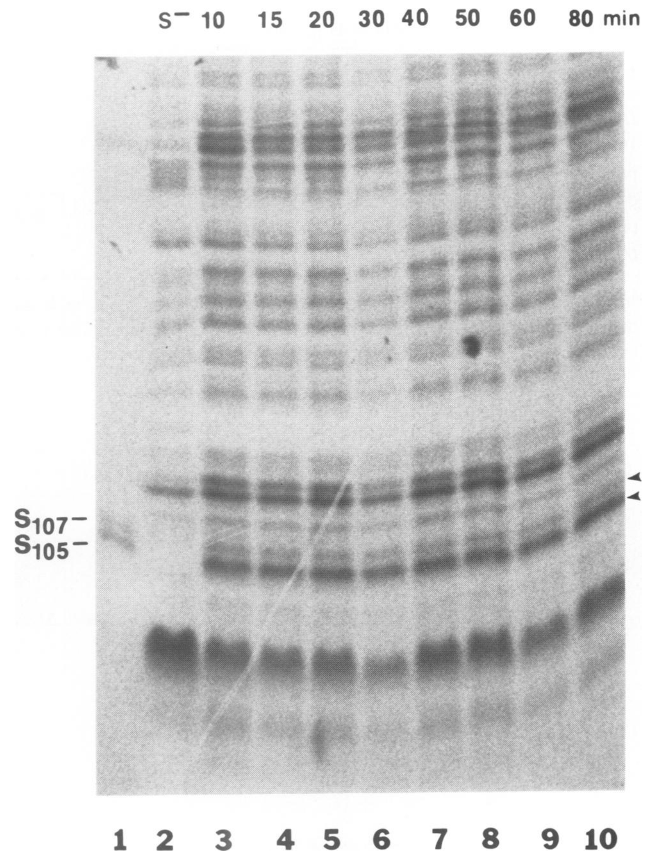

Fig. 4. Stability of proteins S105 and S107. *E.coli* strain MC4100 (pBS125) was grown in minimal medium, infected with λ CE6 and labeled with [^{35}S]methionine as described in Materials and methods. The culture was then chased with unlabeled methionine and further protein synthesis was blocked by addition of chloramphenicol. Samples were removed at various times during the chase and analyzed on a 17.5% SDS–polyacrylamide gel. **Lanes 3–10**, total labeled cellular protein after 10, 15, 20, 30, 40, 50, 60 and 80 min of chase, respectively. **Lane 1**, *in vitro* translated S^+. The positions of the two S polypeptides, S105 and S107, are given at the left and by the two arrows at the right.

that both S proteins are stable over a period of 80 min and that S107 is not degraded or converted to S105.

Protein S107 acts in trans

A model in which pore formation by S105 is disturbed by S107 would require that S107 inhibition works in *trans*. Therefore, compatible plasmids were constructed carrying the S^+ gene under control of the *lac* promoter (pACS) and the S107 allele under control of λ p_R (pGS107; see also Table III). There was no detectable lytic effect when these plasmid borne genes were induced in *E.coli* JM103 (Table I; see also Figure 6A), whereas induction of the S^+ allele in the presence of the parental p_R vector resulted in lysis (Table I; see also Figure 6A).

We next asked whether S107 supplied in *trans* can delay λ lysis. To avoid transcriptional interference with the p_L vectors pLS107 and pLS112 (Tables II and III) carrying the *S107* allele, a λ *imm*^{434} *cts* S^+ phage was utilized as a resident prophage to provide S^+. Compared to the induction of λ *imm*^{434} *cts* S^+ in the presence of the parental vector or plasmid pLS130 (S^- allele), lysis of λ *imm*^{434} *cts* S^+ was delayed (Table I) in the presence of S107 (supplied from plasmid pLS107 or pLS112). It should be noted that a stronger inhibitory effect was observed when S107 was supplied from plasmid pLS112 (Table III), which allows, when compared to pLS107 (Table II), a higher production of S107 (see below). These experiments demonstrate that S107 indeed acts in *trans* and that the degree of inhibition depends on its intracellular concentration.

Charge changes at the amino terminus convert S107 into a lysis effector

S107 and S105 differ only by the Met1–Lys2 residues at the beginning of the longer product. To test whether the inhibitory function of S107 is due to the single positive charge at its amino terminus, Lys2 was converted to Thr by site-directed mutagenesis on an S^+ template. The lysis profiles obtained with the S^+ and *S* (Lys2 → Thr) allele were significantly different (Figure 5). The onset of lysis and the rate of lysis after its inception was faster with plasmid pLS2 (*S*/Lys2 → Thr) than with plasmid pLS157 (S^+). This observation indicates that the S107 inhibition is abolished in this mutant and suggested that the neutral Thr

Table I. Protein S107 inhibits lysis in *trans*

E.coli strain	Plasmids[b]	S alleles	Lysis time[a]
JM103	pACS/pHG276	$S^+/-$	120
JM103	pACS/pGS107	$S^+/S107$	–
pop2135 λ *imm*^{434} *cts* S^+	pLS107	$S^+/S107$	40
pop2135 λ *imm*^{434} *cts* S^+	pLS112	$S^+/S'107$	44
pop2135 λ *imm*^{434} *cts* S^+	pLS130	S^+/S^- (Met1,3 → Leu)	35
pop2135 λ *imm*^{434} *cts* S^+	pLc236	$S^+/-$	35

[a]Cultures were grown at 30°C to an optical density of 0.2 and then shifted to 42°C. Expression of gene *S* from plasmid pACS was induced at the time of temperature shift by addition of isopropyl-β-thiogalactoside to a final concentration of 5 mM. Lysis time is defined as the time in minutes between shift to 42°C and onset of lysis. (–) signifies no lysis.
[b]The plasmids used are described in Tables II and III.

Table II. *E.coli* strains, λ phage and plasmids used in this study

Strain	Genotype/relevant features	Source or reference
Bacterial strains		
MC4100	F$^-$ *Δlac rpsL thi relA*	Raab *et al.* (1988)
JM103	*Δ(lac pro) supE thi hsdR/F′ traD pro*$^+$ *lacI*^{q} *ΔlacZ*$_{M15}$	Messing *et al.* (1981)
CJ236	*dut ung thi relA1* F′::*Tn9*	Kunkel *et al.* (1987)
pop2135	*endA thi hsdR malT (cI857 p*$_R$*)::malPQ*	O.Raibaud, Institute Pasteur, Paris
CSH7	F$^-$ *lacY rpsL thi*	Miller (1972)
Phages		
λ CE6	*Sam7 cI857 int*:: (T7 gene *1*)	Studier and Moffatt (1986)
λ imm^{434}	cts *Sam7*	laboratory stock
Plasmids		
pLc236	λ p_L promoter, amp^R	Remaut *et al.* (1981)
Bluescript pBS$^-$	T3, T7 promoters, amp^R phage f1 origin	Stratagene Cloning Systems
pAC9	*lacPO*, kan^R, P15A origin, derivative of pACYC184	T.O.Baldwin, Texas A&M University, College Station
pHG276	λ p_R promoter, amp^R, λ *cI857* repressor allele	Gordon *et al.* (1986)
pLS157	carries λ S^+, *R* and *Rz* under λ p_L control, amp^R	Bläsi *et al.* (1989)
pLS107	carries the *S107* (Met3 → Leu) allele together with genes *R* and *Rz* under λ p_L control, amp^R	Bläsi *et al.* (1989)
pLS130	carries the S^- (Met1,3 → Leu) allele together with genes *R* and *Rz* under λ p_L control, amp^R	Bläsi *et al.* (1989)
pBS125	carries the λ lysis cassette under T7 control, directs a higher ratio of Met1:Met3 initiations than S^+	Bläsi *et al.* (1989)

residue confers lethal properties on the S107 (Lys2 → Thr) product.

Therefore, Lys2 was converted to Thr using the *S107* (Met3 → Leu) template pBS112, which also carries two single base changes in the left stem of the upstream stem–loop structure (Table IV). This allele (*S′107*) is designed to destabilize the secondary structure, in which the Shine–Dalgarno domain for *S107* is embedded (Bläsi *et al.*, 1989) and thus allows a higher expression of *S107* (data not shown). Induction of the *S′107* (Lys2 → Thr) allele from plasmid pLS21 (Table III) was followed by rapid lysis. Onset of lysis started 10 min earlier than with the S^+ gene (Figure 5). Induction of the control plasmid pLS112 (Table III) carrying the *S′107* allele did not lead to cell lysis (data not shown).

To test the influence of a negatively charged residue in position 2 on the properties of S107, Lys2 was changed to Glu using the *S′107* template, described above. As observed with the *S′107* (Lys2 → Thr) allele, induction of this allele from plasmid pLS22 (Table III) caused cell lysis and onset of lysis was faster than with S^+ (Figure 5).

Energy poisons trigger the lethal potential of S107

Since both changes Lys2 → Thr and Lys2 → Glu converted the inhibitor to a lethal protein, we concluded that the inhibitory function of S107 is entirely due to the positive charge at its amino terminus. Earlier studies have demonstrated that lysis mediated by cloned gene *S* can be triggered prematurely by cyanide and other energy poisons (Garrett and Young, 1982). This suggested that the energized membrane is required for the inhibitory function of S107.

To test whether the addition of cyanide can abolish the S107 lysis block, *E.coli* JM103 cells carrying the compatible plasmids pACS (S^+; Table III) and pGS107 (*S107*; Table III) or the combination pACS/pHG276 (parental vector of pGS107) were grown to an OD_{550} of 0.2, when *S* expression was induced. Premature lysis occurred immediately after addition of the energy poison (Figure 6A), demonstrating that inhibition of lysis effected by the excess of S107 was abolished. Since the rate of lysis after its inception was faster when S107 was additionally supplied in *trans* than with the triggered S^+ gene alone (Figure 6A), we conclude that S107 participates actively in pore formation, i.e. S107 is con-

Table III. Construction of expression vectors carrying the λ lysis cassette

Plasmid	Construction/relevant features
pBS110	insertion of the *SRRz* lysis cassette obtained from plasmid pRG1 (Garrett *et al.*, 1982) into the *Eco*RI–*Hin*dIII sites of pBS$^-$, carries the λ lysis genes under T7 control, used as wild-type template for *in vitro* mutagenesis, amp^R
pACS	insertion of the *SRRz* lysis cassette on a *Sal*I–*Pst*I fragment obtained from pBS110 into pAC9, carries the λ lysis genes under *lac* PO control, kan^R
pGS107	insertion of the λ lysis cassette with the *S107* allele (Met3 → Leu), obtained from pBS109 (Table IV), into the *Eco*RI–*Hin*dIII sites of pHG276, carries the λ lysis genes under λ p_R control, *cI857* allele, amp^R
pLS112	insertion of the λ lysis cassette with the *S'107* (Met3 → Leu) allele, obtained from pBS112 (Table IV), into the *Eco*RI–*Hin*dIII sites of pLc236, carries the *S107* increased-expression allele under λ p_L control, amp^R
pLS2	insertion of the λ lysis cassette with the *S* (Lys2 → Thr) allele, obtained from pBS2 (Table IV), into the *Eco*RI–*Hin*dIII sites of pLc236, amp^R
pLS21	insertion of the λ lysis cassette with the *S'107* (Met3 → Leu; Lys2 → Thr) allele, derived from pBS21 (Table IV), into the *Eco*RI–*Hin*dIII sites of pLc236, amp^R
pLS22	insertion of the λ lysis cassette with the *S'107* (Met3 → Leu; Lys2 → Glu) allele, derived from pBS22 (Table IV), into the *Eco*RI–*Hin*dIII sites of pLc236, amp^R

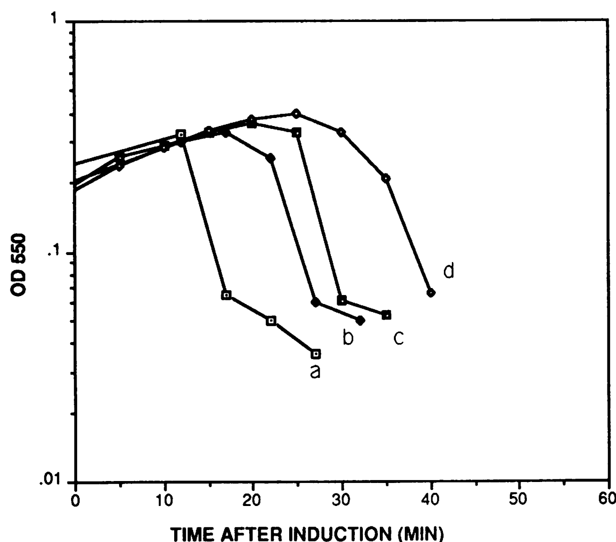

Fig. 5. Lys2 → Thr and Lys2 → Glu changes convert S107 into a lysis effector. For description of the plasmids used, see Tables II and III. *E.coli* strain pop2135 harboring plasmids pLS21 (**a** *S'107*/Lys2 → Thr), pLS2 (**b**; *S*/Lys2 → Thr), pLS22 (**c**; *S'107*/Lys2 → Glu) and pLS157 (**d**; S^+), respectively, was grown at 30°C in LB broth supplemented with 100 μg/ml ampicillin to OD_{550} = 0.2. At time 0, the different *S* alleles were induced by shifting the cultures to 42°C.

verted to a lysis effector once the membrane potential is dissipated.

The results in Figure 6B confirm this directly. The cloned S107 protein can be triggered with cyanide in the absence of S105. Using the *S'107* allele, which supports increased synthesis of S107, causes even faster lysis after triggering by cyanide (Figure 6B). Cells carrying the control plasmid pLS130 (S^-) did not lyse, demonstrating the S107 dependency of the cyanide-induced lysis. These results demonstrated that not only is the inhibitory function of S107 abolished by cyanide but also that the collapse of the membrane potential triggers the lethal potential of S107.

Discussion

sdi model for *S* gene expression confirmed *in vivo*

Previously, we proposed a model for *S* gene function and regulation, derived from genetic analysis of a collection of *S* mutants isolated as lysis defective alleles or constructed by site-directed mutagenesis (Raab *et al.*, 1988; Bläsi *et al.*, 1989). According to the model, translational initiations occur at the start codons Met1 and Met3 leading to synthesis of an S107 and an S105 product, respectively. Here, we have shown that, both *in vivo* and *in vitro*, two polypeptides are synthesized from the *S* gene (Figures 2 and 3). In addition, the difference in mass, as estimated by SDS–PAGE, and net charge, as apparent from non-equilibrium isoelectric focusing, is consistent with the predicted primary structure of the two products. Moreover, our previous toeprinting analysis showed that initiations at Met3 and Met1 occur with a ratio of 2.5:1, in spite of the fact that only Met1 had a canonical Shine–Dalgarno sequence with the appropriate spacing (Bläsi *et al.*, 1989). This was shown to be due to features of the primary and secondary structure of the 5′ region of the *S* gene, here redesignated as the *sdi* locus. Here we have shown that, *in vivo*, the smaller and more acidic product, S105, is synthesized in significant excess over S107, in fact in even greater excess than *in vitro* (Figures 2 and 3).

Model and molecular basis of S107 lysis inhibition

Since the ratio of Met1:Met3 initiations *in vitro* could be correlated with the time required for lysis using cloned *S* alleles, we have postulated that S107 acts as an inhibitor of the S105 function (Bläsi *et al.*, 1989). Here, we demonstrated that S107 supplied in *trans* can inhibit S105-induced lysis, and that the degree of inhibition can be correlated with the relative production of the two forms of S (Table I). In a situation in which the S107 product is produced from the strong p_R promoter on a high copy number plasmid, while the S^+ gene is expressed from the weaker *lac* promoter on a lower copy number plasmid, lysis is completely abolished (Table I; Figure 6A). Thus, the hypothesis that S107 is a lysis inhibitor can be regarded as proven.

How does S107 act to inhibit the lysis effector S105? Since the two polypeptides differ only by the two amino-terminal amino acids, it was not unexpected to find that the operative distinction is the Lys2 residue. To account for the inhibitory properties of this single positively charged residue consistent with the stable, membrane-bound character of both proteins (Figure 3), we propose a two-step model for the formation of the S pore. We suggest that after induction of *S* gene expression, S105 and S107 molecules accumulate as a hetero-oligomeric patch in the inner membrane and then, in a second step, rearrange to form the S pore (Figure 7B). This rearrangement is inhibited by the interaction of the amino-terminal positive charge of the S107 molecules with the energized membrane. The simplest rationale is that the positively charged Lys2 residue of S107 has an ionic

Table IV. Construction of mutant *S* alleles by *in vitro* mutagenesis

Mutagenic oligonucleotide[a]	Template	Mutation(s)	Plasmid
3'-CATTCTGTACTGCTACGGTC-5'	pBS110	Lys2 → Thr	pBS2
3'-ATTCTGTACTGCGACGGTCTT-5'	pBS112	−17C → G; −19C → G; Lys2 → Thr; Met3 → Leu	pBS21
3'-ATTCTGTACCTTGACGGTCTTT-5'	pBS112	−17C → G; −19C → G; Lys2 → Glu; Met3 → Leu	pBS22
3'-CATTCTGGACTTCTACGGTCTT-5'	pBS110	Met1 → Leu	pBS108
3'-CATTCTGTACTTCGACGGTCTT-5'	pBS110	Met3 → Leu	pBS109
3'-CTCGTTTAGCGCAATAACCCCC-5'	pBS109	−17C → G; 19C → G; Met3 → Leu	pBS112
3'-CATTCTGGACTTCGACGGTCTT-5'	pBS110	Met1,3 → Leu	pBS122

[a]Mismatches with the template sequence are underlined.

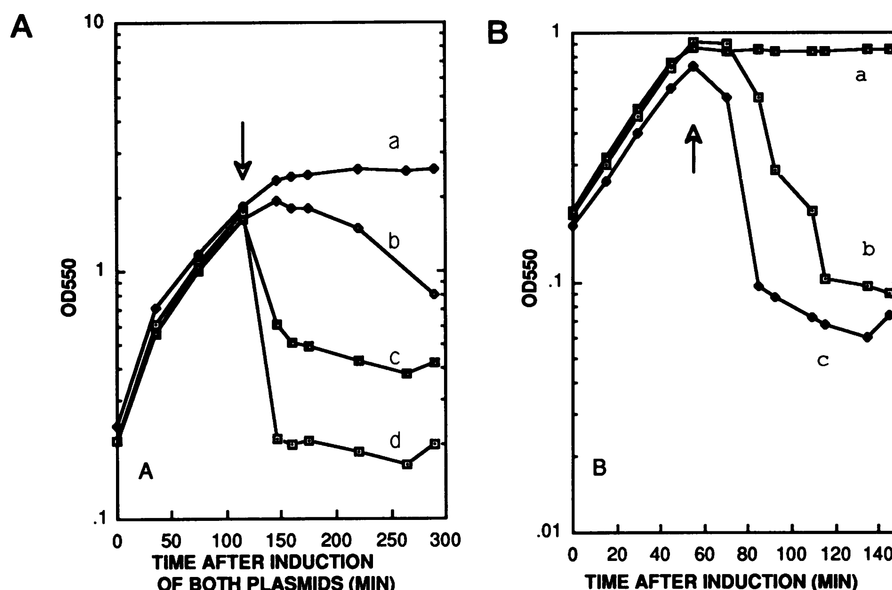

Fig. 6. Cyanide triggers the lethal potential of S107. For description of the plasmids used, see Tables II and III. (**A**) The S107 lysis inhibition in *trans* is abolished by cyanide. *E.coli* strain JM103 carrying the plasmid pairs pACS/pGS107 (a and d) and pACS/pHG276 (b and c), respectively, was grown in LB broth supplemented with 50 μg/ml kanamycin and 100 μg/ml ampicillin to OD_{550} = 0.2 (time 0), when the cultures were shifted to 42°C (induction of *S107* from plasmid pGS107) and isopropyl-β-thiogalactoside (induction of S^+ from plasmid pACS) was added to a final concentration of 5 mM. At the time indicated by an arrow KCN was added to a final concentration of 10 mM to cultures (c) and (d). (**B**) S107 causes lysis after dissipation of the membrane potential. *E.coli* strain pop2135 carrying the plasmids pLS130 (a; S^-), pLS107 (b; *S107*) and pLS112 (c; *S'107*) was grown and induced as described in (A). At the time indicated by an arrow KCN was added to a final concentration of 10 mM.

interaction with the negatively charged inner surface of the energized membrane (Figure 7A). This ionic interaction would serve to anchor the S107 molecules against lateral diffusion. We expect the membrane-spanning domains of the S107 and S105 molecules to be identically disposed within the bilayer and to interact both intra- and inter-molecularly. Thus, the anchoring of the S107 molecules would also retard the lateral diffusion of the S105 molecules and consequently inhibit pore formation. In this model, the lysis antagonist S107 exerts its inhibitory effect primarily in the rearrangement step. Alternatively, S107 may differ from S105 in some aspect of its disposition within the bilayer. However, such differences must be subtle, since the two *S* products are both found to be stable membrane proteins (Figures 3 and 4). The model is directly supported by the phenotypes associated with the Lys2 → Thr and Lys2 → Glu mutations. Both changes would abolish the proposed interaction with the energized membrane and therefore allow S107 to act as a lethal lysis effector (Figure 5). The type of ionic interaction proposed for the amino terminus of the S107 polypeptide has precedence in the 'loop' model of Inouye *et al.* (1982), proposed as a first step in signal sequence recognition. In that case, the amino-terminal positive charge is essential for proper signal sequence cleavage.

What triggers the lytic event and the rearrangement of non-functional S105/S107 hetero-oligomeric patches? It may be that the first pore forms simply by a stochastic process, the kinetics of which are determined by both the absolute concentration of total S protein and the proportion of S105 within the population. The formation of the first pore in a patch particularly rich in S105 monomers would rapidly disrupt the membrane potential, thereby reducing the ionic interaction which anchors the amino-terminal domains of the S107 molecules to the inner surface of the cytoplasmic membrane and thus allowing rapid rearrangement and pore formation within the other S105/S107 hetero-oligomeric patches. This model is supported by the data in Figure 6 which indicate that S107 molecules participate in pore formation. Thus, the model contains a natural saltatory mechanism which is attractive in view of the very sharp lysis profiles characteristic of induced λ lysogens (Campbell and Rolfe, 1975). In addition, it suggests a molecular basis to the phenomenon of 'premature lysis', one of the first physiological characteristics noted for λ-infected cells

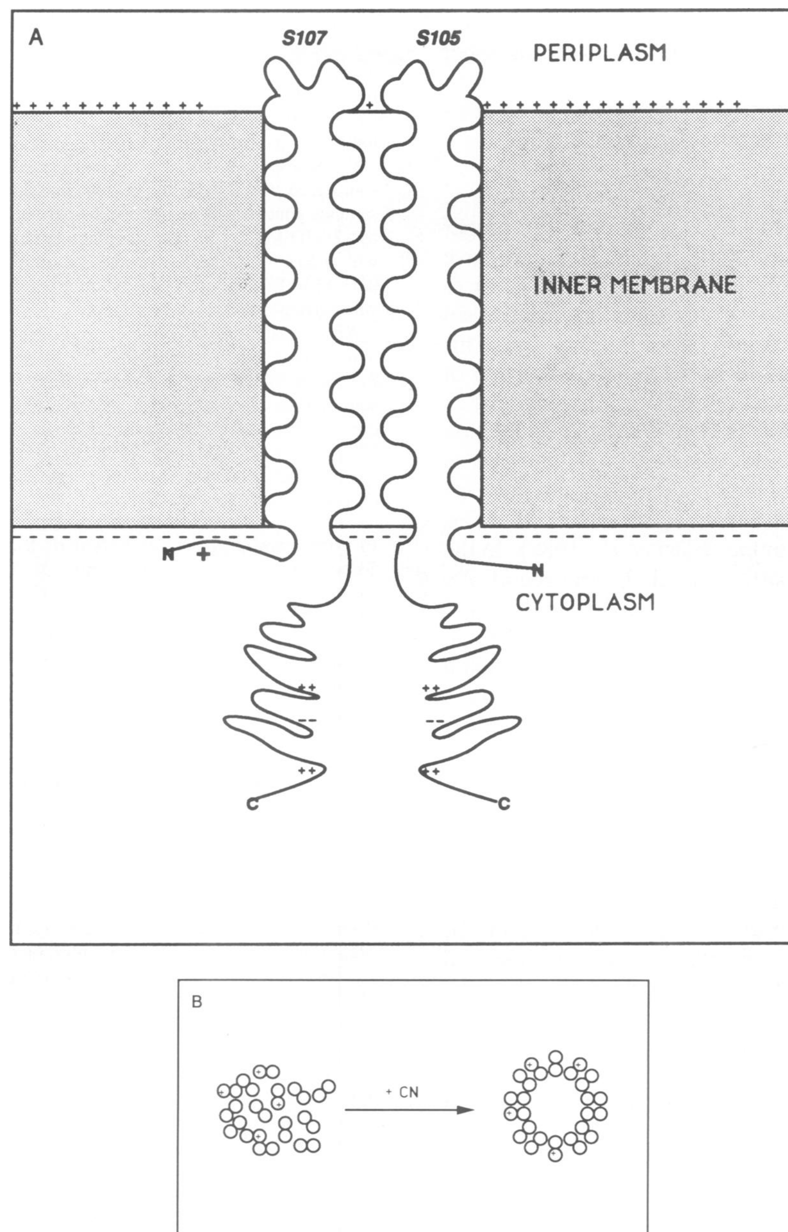

Fig. 7. Model of S107 inhibition. (**A**) S105/S107 hetero-oligomers form in the inner membrane. For clarity only two S molecules are depicted. The positively charged Lys2 residue of S107 interacts with the negatively charged surface of the inner membrane and thereby restricts lateral diffusion of S105/S107 hetero-oligomers and inhibits pore formation. (**B**) Dissipation of the membrane potential by energy poisons or by formation of a functional S pore (i.e. abolishing the ionic interaction between the Lys2 residue of S107 and the inner membrane) allows rearrangement of the S105/S107 molecules organized in mixed oligomeric patches. Rearrangemement by lateral diffusion within the patch then results in pore formation. ⊕, S107 molecules; ○, S105 molecules.

(Reader and Siminovitch, 1971b). Addition of energy poisons causes cells supporting the late vegetative phase of λ growth to lyse immediately if S is functional (Reader and Siminovitch, 1971b). We have shown here that the absolute block in lysis caused by an excess of S107 can be instantaneously subverted by KCN (Figure 6A). Also, as shown in Figure 6B, KCN can trigger the lethal potential of S107. Thus, 'premature lysis' observed after cyanide treatment of infected cells and the collapse of the S107-dependent lysis block (i.e. conversion of the non-lethal lysis inhibitor S107 into an active pore component) are one and the same event.

This model fundamentally requires that S molecules oligomerize, and there are several lines of evidence to support this notion. S oligomers up to a tetramer size have been detected in the inner membrane of *E.coli* by cross-linking and SDS-resistant dimers were visualized when purified S protein was subjected to SDS−PAGE (Zagotta and Wilson, 1990). Also, preliminary results suggest that S oligomers can form in yeast plasma membranes (C.Bruno, J.Garrett and R.Young, unpublished). Furthermore, genetic analysis revealed that about one-third of a collection of defective *S* alleles are, at least, co-dominant, and the pattern of dominants and recessives suggested that the second putative membrane spanning region, between residues 40 and 60, is required for oligomerization (Raab *et al.*, 1988). All these data suggest that protein S has a strong tendency to oligomerize, and since no form of S has ever been detected

in the soluble fraction, we have constructed the simplest model by having S oligomers form directly in the inner membrane (Figure 7A). To test our proposed model, experiments are now under way to provide direct biochemical evidence for the S105/S107 interaction.

Regulation of lysis

We have previously shown that S107 is important for proper scheduling of cell lysis (Bläsi *et al.*, 1989). Here, we have provided evidence that S107 not only acts to delay or to inhibit lysis but that it is also part of a saltatory mechanism which is inherent to the lysis control mechanism. This strategy would facilitate a sudden and dramatic bursting of the cell and thus efficient release of phage progeny. Is this model for the molecular basis of the S105/S107 interaction sufficient to explain how lysis is regulated? We have shown that the structural features of the *sdi* locus are set to generate 2.5:1 partition of initiation events at Met3 and Met1, as measured *in vitro* by toeprinting (Bläsi *et al.*, 1989). In the simplest formulation, this partition might be realized *in vivo* throughout the late transcription period, giving an excess of S105 over S107 which could lead, as mentioned earlier, to the formation of a pore in a stochastic manner. It should be noted that this mechanism would serve to ensure a saltatory triggering of lysis within a single cell; however, within a population of cells, one might expect a broad distribution in the times required to reach the trigger point. Several lines of evidence suggest that there is at least one more level of regulation in the control of lysis. First, the observed ratio of S105:S107 production *in vivo* (Figure 3) is significantly higher than predicted from toeprint analysis (Bläsi *et al.*, 1989) or from *in vitro* translation (Figure 2), suggesting that, *in vivo*, a regulatory factor may be involved in altering the partition of initiation events. Logically, one might expect a shift toward S105 production as the late protein period progresses. Preliminary results employing the T7 expression system suggest that, in fact, S107 production predominates early after induction of *S* gene expression, and then S105 synthesis dominates after ~30–40 min of synthesis (C.-Y.Chang, U.Bläsi and R.Young, unpublished data). In addition, in work to be described elsewhere, we have isolated an external suppressor of a defective *S* allele, also suggesting that at least one other λ gene participates in modulation of *S* gene expression.

Materials and methods

Strains and plasmids

A list of *E.coli* strains and plasmids used is given in Table II. If not otherwise indicated, cultures were grown in Luria broth (Miller, 1972) supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml) if a plasmid was present. Growth and lysis profiles were monitored by measuring the OD_{550}.

Construction of λ imm^{434} cts S^+

Dilutions of a λ imm^{434} *cts Sam* lysate were spotted on a lawn of *E.coli* MC4100 (pRG1). Plasmid pRG1 (Raab *et al.*, 1986) carries the entire λ lysis gene region, consisting of genes *S*, *R* and *Rz*. Prospective λ imm^{434} *cts* S^+ recombinants were judged by plaque size and further purified on MC4100. Lysogens were constructed by infecting *E.coli* pop2135 with a m.o.i. ~0.1 and selected by cross-streaking with λ imm^{434} cI^-. Strain pop2135 carries a chromosomal copy of the thermosensitive allele of the phage λ *cI* repressor gene. This system avoids transcriptional interference with the p_L expression vectors from which the S107 was supplied in *trans*.

NEPHGE and Western blotting

NEPHGE was carried out as described by O'Farrell *et al.* (1977), with the following modifications. A slab gel consisting of 8 M urea, 4% acrylamide (28.4% acrylamide:1.5% bis-acrylamide), 2% Triton X-100, 2% ampholines was used. The two forms of λ S protein could be separated using equal volumes of pH 3–10 and pH 6–9 ampholines. Crude membrane fractions were prepared from cultures of induced CSH7 λ S^+R^- and CSH7 λ S^-R^- lysogens, respectively. The cells were harvested 65 min after induction, passed through a French pressure cell and ultracentrifuged at 100 000 *g* for 1 h at 4°C to pellet the membranes. The pellets were then solubilized with 1% Triton X-100, and additions were made so that the samples contained 8 M urea, 0.3 M β-mercaptoethanol, 2% ampholines and 2% Triton X-100. The samples were loaded onto the gel, and overlaid with a solution of 8 M urea and 2% ampholines. The anode buffer was 0.01 M H_3PO_4 and the cathode buffer was 0.02 M NaOH. Electrophoresis was carried out for 4 h at 400 V.

After separation, the proteins were electrophoretically transferred to a nitrocellulose sheet (pore size 0.1 μm), as described by Towbin *et al.* (1979). For the NEPHGE gels, the transfer buffer was composed of 0.7% acetic acid and the electrodes were in opposite orientation as compared to the transfer direction of SDS–polyacrylamide gels. The filter was blocked with 3% gelatin in 20 mM Tris–HCl, pH 7.5, and 150 mM NaCl. S protein-specific bands were detected using preadsorbed anti-S antibodies as described (Zagotta, 1989).

In vitro transcription/translation of S alleles

Samples of 5 μg of each plasmid, pBS108 (*S105*), pBS109 (*S107*), pBS110 (S^+) and pBS122 (S^-) carrying the different *S* alleles together with the accessory lysis genes *R* and *Rz* under control of a T7 promoter (see Tables III and IV) were first cut with *Hin*dIII. *In vitro* transcription of the *S*, *R* and *Rz* gene region was carried out with T7 polymerase using the Riboprobe Gemini System II (Promega Biotec) according to the manufacturer's instructions. The T7 derived run off transcripts were translated *in vitro* using the methodology described by Zubay (1980).

In vivo expression of S alleles with the T7 system

E.coli strain MC4100 harboring plasmids pBS108, pBS109, pBS110 and pBS122, respectively (Tables III and IV), were grown at 30°C in M9 maltose medium (Miller, 1972; Studier and Moffatt, 1986). At an OD_{550} of 0.3, glucose was added to a final concentration of 4 mg/ml. Aliquots of 5 ml of the cultures were shifted to 37°C and incubated for a further 90 min. Prior to infection with λ phage CE6 (Table II) at a multiplicity of 10, $MgSO_4$ was added to a final concentration of 10 mM. After a pre-adsorption period of 30 min at 37°C, rifampicin was added to a final concentration of 15 μg/ml. After 30 min, the cells were labeled with [^{35}S]methionine for 10 min at 37°C. Then the cells were centrifuged, washed with washing buffer (10 mM $MgCl_2$, 10 mM MOPS, pH 7.5), resuspended in 1 ml of washing buffer and passed through a French pressure cell. Intact cells were removed by low speed centrifugation and the supernatant was ultracentrifuged at 100 000 *g* for 1 h. The pellet containing the membrane fraction was washed twice with washing buffer and resuspended in SDS sample buffer (Laemmli, 1970). The cytoplasmic proteins were precipitated from the supernatant after the ultracentrifugation step by addition of an equal volume of 10% trichloroacetic acid (TCA). The pellet was washed twice with acetone and resuspended in SDS sample buffer.

Pulse–chase experiments

A 5 ml culture of *E.coli* MC4100 carrying plasmid pBS125 (Table II) was grown in M9 maltose medium and infected with λ phage CE6 exactly as described above. At 30 min after infection the cells were labeled with 100 μCi [^{35}S]methionine for 10 min, a 200 μl sample was removed, and radioactive incorporation was terminated by adding 4.8 ml prewarmed M9 medium containing 2% unlabeled methionine and 300 μg/ml chloramphenicol. Samples of 200 μl were removed at various times during the chase, the proteins were immediately precipitated by addition of TCA to a final concentration of 5%. Finally, the samples were resuspended in SDS sample buffer.

SDS–PAGE

To separate the two forms of protein S, which differ only by two amino acids, samples were run on a 40 cm SDS–polyacrylamide gel for 8500 V h. The gel composition was basically as described by Laemmli (1970), except that the Tris concentration was doubled in the separating gel and in the running buffer (Fling and Gregerson, 1986). The [^{35}S]methionine-labeled proteins were visualized by autoradiography.

Site-directed mutagenesis

Site-directed mutagenesis was carried out as described by Kunkel *et al.* (1987). The single-stranded templates were prepared with the helper phage M13KO7 from *E.coli* strain CJ236 (Table II) transformed with different pBS^- vectors (Tables III and IV). Basically, the pBS^- vectors carried the

*Eco*RI–*Hin*dIII fragment of plasmid pRGI (Raab *et al.*, 1987), which contains the region of the λ genome from the *Eco*RI site at 44972 to the *Cla*I site at 46438, a segment which spans the three lysis cistrons *S*, *R* and *Rz*. Candidate clones were sequenced as described earlier (Raab *et al.*, 1986). The mutagenic oligonucleotides employed on the different templates and the corresponding mutations generated are given in Table IV.

Cloning of different S alleles in expression vectors

To demonstrate the phenotypical effect of the *in vitro* mutated *S* alleles, the *SRRz* genes were reisolated on an *Eco*RI–*Hin*dIII cassette and inserted by standard methods (Maniatis *et al.*, 1982) into the p_L expression vector pLc236 (see Tables II and III). Furthermore, the *S107* allele was cloned together with *R* and *Rz* under λ p_R control into the expression vector pHG276 and the 'wild-type *SRRz* cassette' was inserted under control of the *lac* promoter in plasmid pAC9, which contains the P15A origin (see Tables II and III).

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