JOURNAL OF BACTERIOLOGY, Feb. 1993, p. 1038–1042
0021-9193/93/041038-05$02.00/0

Vol. 175, No. 4

The Missing Link in Phage Lysis of Gram-Positive Bacteria: Gene *14* of *Bacillus subtilis* Phage φ29 Encodes the Functional Homolog of Lambda S Protein

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Received 11 September 1992/Accepted 5 December 1992

In most bacteriophages of gram-negative bacteria, the phage endolysin is released to its murein substrate through a lesion in the inner membrane. The lesion is brought about by a second phage-encoded lysis function. For the first time, we present evidence that the same strategy is elaborated by a phage of a gram-positive bacterium. Thus, there appears to be an evolutionarily conserved lysis pathway for most phages whether their host bacterium is gram negative or gram positive. Phage φ29 gene *14*, the product of which is required for efficient lysis of *Bacillus subtilis*, was cloned in *Escherichia coli*. Production of protein 14 in *E. coli* resulted in cell death, whereas production of protein 14 concomitantly with the φ29 lysozyme or unrelated murein-degrading enzymes led to lysis, suggesting that membrane-bound protein 14 induces a nonspecific lesion in the cytoplasmic membrane.

It appears to be a general phenomenon that bacteriophages of gram-negative bacteria encoding an endolysin also code for a second lysis function which permits access of the endolysin to its murein substrate. Apparently, the requirement for a second lysis function is due to the fact that none of the characterized phage-specific endolysins (1, 10, 22, 28) possess a signal sequence for transit across the inner membrane.

The best-studied lysis system in this phage class, the lambda lysis operon, encompasses three genes designated *S*, *R*, and *Rz* (8). The function of the *Rz* gene is unknown. The *R* gene encodes the peptidoglycan-degrading enzyme of the lambda lysis cassette (1). Its release to the periplasm strictly depends on protein S. It has been shown that S monomers oligomerize in the inner membrane (32), resulting in formation of a nonspecific lesion with undefined dimensions (30). Essentially the same lysis system is operational in the *Salmonella typhimurium* phage P22 (18, 22) and the *Escherichia coli* phage 21 (5). Furthermore, genes *17.5*, *t*, and *P10* of the *E. coli* phages T7 (31) and T4 (17, 23) and the *Pseudomonas phaseolicola* phage φ6 (16), respectively, appear to serve a lambda *S*-like function.

Some murein hydrolase genes of phages of gram-positive bacteria have been sequenced: gene *15* of the closely related *Bacillus subtilis* phage φ29 and PZA (10, 19, 29), and a gene termed lysin from the *Lactococcus lactis* phage φvML3 (25). The φ29 and φvML3 lysozymes have both been overproduced in *E. coli* (24, 25) without exerting a toxic effect. Consistent with this observation, no signal sequence is predicted for either the gene 15 product or the φvML3 lysin. Although it seems widely accepted that for gram-positive bacteria only a phage endolysin is required for host cell lysis, the question arises of how a murein hydrolase activity could possibly affect the cell envelope while being localized in the cytoplasm. Hints of the presence of a second lysis function come from studies of the φ29 *sus14* (1242) mutant. When grown on a nonsuppressing strain, the phage mutant showed a delay in lysis time and an increase in burst size (6). These observations and the fact that gene *14* maps immediately upstream of gene *15* (10), at a position analogous to those of lambda gene *S* and the P22 gene *13*, prompted us to test whether φ29 gene *14* may encode a lysis function required for efficient release of the phage lysozyme to the murein.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. *E. coli* MC4100I^{Q} *lac araD U169* Sm^{r} *thi relA* (F′ *proAB lacI*^{q} *ZΔM15* Tn*10*) was constructed by mating the F′ from strain XL1-Blue (Stratagene Cloning Systems) into strain MC4100. *B. subtilis* 110NA *su*$^{-}$ and MO-101-P *spoA thr su*$^{+44}$, as well as φ29 wild type and φ29 *sus14* (1242), were kindly provided by M. Salas, University of Madrid. Lambda phage CE6 *Sam*7 *c*I857 *int*::(T7 gene 1) (27), as well as plasmids pKS^{-} (Stratagene Cloning Systems), pK194 (13), pLysS (26), and pJG16 (9), have been described elsewhere.

Construction of plasmids bearing φ29 genes *14* and *15*. φ29 and φ29 *sus14* (1242) double-stranded DNA was prepared and digested with proteinase K, as described before (12). The DNA region comprising genes *14* and *15* was obtained from both phage DNAs by amplification, using the polymerase chain reaction and the synthetic oligonucleotides A (5′-CTTTTACTATCTGCAGCGCCC-3′) and B (5′-GC GATATCAAAATCAACTTAATCTAATTGTTTGACC-3′). Oligonucleotide A anneals to base pairs −57 to −38 upstream of the start codon of gene *14*. It was designed to create a *Pst*I restriction site at position −44 relative to oligonucleotide A of the gene *14* AUG start codon. Oligonucleotide B is complementary to the last eight codons of gene *15*. The 12 protruding bases at its 5′ end contain an *Eco*RV site. Both primers were annealed to either φ29 or φ29 *sus14* (1242) DNA and extended with *Pyrococcus furiosus* polymerase (Stratagene Cloning Systems), using a standard polymerase chain reaction protocol (14). The resulting 1,244-bp fragments were gel purified and subsequently digested with *Pst*I and *Eco*RV. The *Pst*I-*Eco*RV *14/15*- and *sus14/15*-containing fragments were inserted into the *Pst*I

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TABLE 1. Loss of viability after induction of gene *14*

Gene *14*[a]	Time after induction (min)[b]	% Survival[c]
+	0	100
+	20	10
+	40	2
+	60	0.7
−	0	100
−	20	130
−	40	210
−	60	250

[a] *E. coli* MC4100I^Q harboring plasmid pSB29-11 carrying gene *14* (+) or plasmid pSB29-22 bearing the *sus14* allele (−) was grown at 37°C in LB broth (15) to an optical density at 600 nm of 0.30, when expression of φ29 genes was induced by the addition of IPTG to a final concentration of 5 mM.

[b] Samples (100 μl) were withdrawn from the induced cultures at the times given.

[c] Serial dilutions of the withdrawn samples were plated onto LB plates containing 50 μg of kanamycin per ml. CFU at time zero were taken as 100%, and percent survival was calculated relative to CFU at time zero.

and *Eco*RV sites of plasmid pKS$^-$. The resulting plasmids, pMS29-1 and pMS29-2, carry the φ29 genes *14/15* and *sus14/15*, respectively, under transcriptional control of a T7 promoter.

To place both gene arrangements under control of the *lac* promoter, the *Pst*I-*Eco*RV-digested polymerase chain reaction fragments were individually cloned into the *Pst*I-*Sma*I sites of plasmid pK194, resulting in plasmids pSB29-1 (genes *14/15*) and pSB29-2 (genes *sus14/15*).

φ29 gene *15* was deleted in plasmids pSB29-1 and pSB29-2 by digestion of plasmid DNA with *Eco*RI and subsequent religation. This manipulation eliminated the last 145 codons of gene 15 (10). The resulting plasmids, pSB29-11 and pSB29-22, bear only the intact φ29 gene *14* and the *sus14* allele, respectively.

Expression of φ29 gene *14* in vitro and in vivo. Samples of 5 μg of plasmid pMS29-1 were first digested with *Eco*RI and then transcribed by using T7 RNA polymerase and Riboprobe Gemini System II (Promega Biotec) in accordance with the manufacturer's instructions. The T7-derived runoff transcripts were translated in vitro, as described previously (2).

The growth conditions, infection with lambda CE6, and labelling with ^{35}S-methionine, as well as preparation of the membrane fractions and cytoplasmic/periplasmic fractions of *E. coli* MC4100 harboring plasmid pMS29-1 or pMS29-2, were performed as described recently (2). Samples were electrophoresed on a high-resolution sodium dodecyl sulfate–17.5% polyacrylamide gel as reported before (2).

RESULTS AND DISCUSSION

Expression of φ29 gene *14* is lethal for *E. coli*. Although the 131-amino-acid protein 14 shows no homology with the best-studied, nonspecific, hole-forming protein S of phage lambda, both have a basic isoelectric point and a highly charged C terminus, are highly hydrophobic (10, 20), and share the following striking structural feature: when subjected to computer analysis, protein 14, like protein S, shows a high probability to have a pair of transmembrane domains separated by a beta turn (Fig. 1). On comparative grounds these features are also typical traits for the pore-forming protein 13 of *S. typhimurium* phage P22 (22), phage 21 protein S (5), and the T7 gene product *17.5* (32). The structural similarities together with the fact that protein S functions in *Saccharomyces cerevisiae* (7) suggested that protein 14, like protein S, might be capable of spontaneous integration into lipid bilayers and thus might function in *E. coli*. It should be noted that all of our attempts to clone and express φ29 gene *14* directly in *B. subtilis* failed, which was presumably because of its severe toxicity. Another consideration for using *E. coli*, therefore, was the availability of plasmids containing highly repressible promoters used previously for controlled expression of lambda and φX174 lysis genes (4, 9).

If protein 14 is capable of inducing a nonspecific lesion in the inner membrane of *E. coli*, its expression could dissipate the membrane potential and therefore be lethal. Compared with expression of the *sus14* allele from plasmid pSB29-22, induction of gene *14* from plasmid pSB29-11 was deleterious to *E. coli* MC4100I^Q. Growth arrest was observed 40 min after induction of gene *14* (not shown). Cell viability was measured at 20-min intervals after induction of gene *14* and the *sus14* allele from cultures of MC4100I^Q harboring the respective plasmids by diluting culture aliquots in LB broth and plating onto LB plates containing kanamycin. As shown in Table 1, the cell viability decreased very rapidly after induction of gene *14* in strain MC4100I^Q(pSB29-11), while growth of culture MC4100I^Q(pSB29-22) continued unabated. These results demonstrated that φ29 gene *14* behaves exactly like analagous lambda *S* clones (9) in the absence of the R transglycosylase.

Subcellular localization of φ29 protein 14. To determine the subcellular localization of φ29 protein 14 directly, we employed an in vivo T7 expression system. Since plasmids pMS29-1 and pMS29-2 bear gene *14* and the *sus14* allele, respectively, under transcriptional control of a T7 promoter, they can be expressed after infection with lambda phage CE6, which carries T7 gene *1*. As shown in Fig. 2, lane 2, two protein 14-specific bands with an M_r of approximately 14,000 were detected exclusively in the membrane fraction of MC4100(pMS29-1). Protein 14 was not visible in either the cytoplasmic/periplasmic fraction of the same transformants (Fig. 2, lane 3) or in membrane (Fig. 2, lane 4) or cytoplasmic/periplasmic samples (Fig. 2, lane 5) of strain MC4100 harboring plasmid pMS29-2, which carries the *sus14* allele.

Why does expression of φ29 gene *14* result in two protein species? φ29 gene *14* has in common with lambda gene *S*, P22 gene *13*, and the *S* gene of phage 21 two potential start codons (Fig. 1), both of which have been shown to be utilized by the latter three lysis genes (3, 5, 18). In lambda, the two S proteins have opposing functions in lysis (2, 3). The 107-amino-acid polypeptide starting at Met_1 of *S* acts as a nonlethal inhibitor of S105, whereas the S105 protein starting at Met_3 of *S* can be regarded as the actual lysis effector (2, 21). The same molecular lysis control system has been shown to be conserved in the closely related gene *13* of phage P22 (18) and the functional homolog of *S* in phage 21 (5). It has been proposed that this lysis control mechanism may serve for proper scheduling of cell lysis (3, 21). Similarly to the lysis systems mentioned above, the two protein 14-specific bands shown in Fig. 2, lane 2, could result from initiation events at Met codons 1 and 3 of gene *14*. Hence, it is intriguing to speculate that they might correspond to protein 14 species consisting of 131 and 129 amino acids (Fig. 1), respectively.

φ29 lysis genes function in *E. coli*. On the basis of expression studies of gene *14* (Table 1) together with the fact that protein 14 is membrane bound, we concluded that gene *14* has the same function in lysis as lambda *S*, i.e., induction of

FIG. 1. Primary sequence of protein 14 and predicted transmembrane segments. For comparison, the primary sequence of lambda protein S is also depicted below the protein 14 sequence. Predicted transmembrane segments in both proteins are shown by bars below the sequence. The third putative membrane-spanning domain in lambda S is questionable and indicated by colons. Potential β-turn regions are indicated by t's. Charged amino acid residues are signified by the + and − signs. The beginning of lambda S107 and S105 proteins (2) as well as the beginning of the putative 14-131 and 14-129 proteins are depicted.

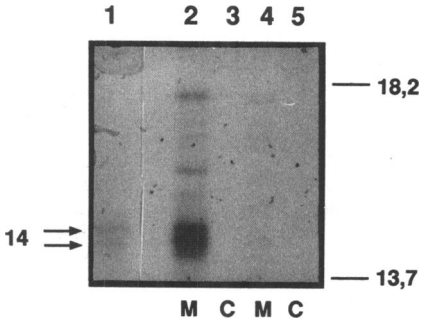

FIG. 2. Membrane localization of protein 14. *E. coli* MC4100 harboring plasmid pMS29-1 (*14+*) or pMS29-2 (*sus14*) was grown in minimal medium (15), infected with lambda CE6, and labelled with ^{35}S-methionine as described before (2). The cytoplasmic/periplasmic fraction (C) and the membrane fraction (M) were isolated, and the samples were analyzed on a sodium dodecyl sulfate–17.5% polyacrylamide gel. The position of protein 14 (lanes 1 and 2) is given by arrows at the left. The positions of marker proteins (molecular masses given in kilodaltons) are indicated at the right. Lane 1, in vitro-translated protein 14; lanes 2 and 3, membrane and cytoplasmic/periplasmic fractions of MC4100(pMS29-1); lanes 4 and 5, membrane and cytoplasmic/periplasmic fractions of MC4100 (pMS29-2).

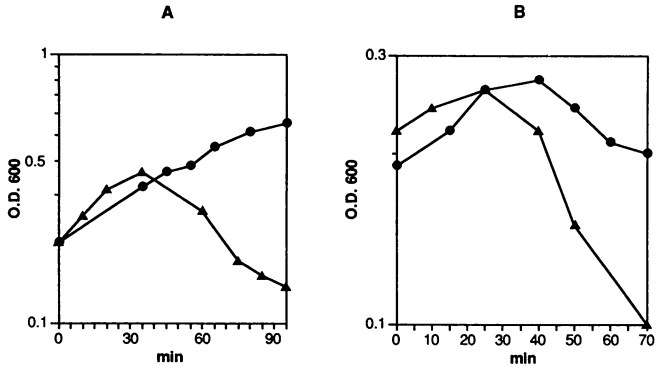

FIG. 3. ϕ29 protein 14 functions in *E. coli*. (A) Expression of genes *14/15* and *sus14/15* in *E. coli*. *E. coli* MC4100I^{Q} harboring plasmid pSB29-1 (▲; *14/15*) or pSB29-2 (●; *sus14/15*) was grown at 37°C in LB broth (15) supplemented with 50 μg of kanamycin per ml. At time zero, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 5 mM. (B) Release of lambda R transglycosylase and the T7 amidase by ϕ29 protein 14. *E. coli* MC4100I^{Q} harboring plasmid pSB29-11 (ϕ29 *14/15−*) or pJG16 (lambda *R*) (▲) was grown at 37°C in LB broth (15) supplemented with 50 μg of kanamycin and 100 μg of ampicillin per ml. At time zero, expression of genes *14* and *R* was induced by addition of IPTG (final concentration, 5 mM). *E. coli* MC4100I^{Q} bearing plasmids pSB29-11 and pLysS (T7 *3.5*) (●) was grown at 37°C in LB broth in the presence of 50 μg of kanamycin and 50 μg of chloramphenicol per ml. Expression of gene *14* from plasmid pSB29-1 was induced as described above. Expression of T7 gene *3.5* was constitutive. O.D. 600, optical density at 600 nm.

a nonspecific lesion in the inner membrane. Therefore, we asked whether expression of gene *14* is required to permit access of the ϕ29 lysozyme to the peptidoglycan layer. Expression of genes *14* and *15* from plasmid pSB29-1 resulted in lysis 40 min after induction (Fig. 3A), whereas no lysis was obtained after induction of plasmid pSB29-2 bearing ϕ29 genes *sus14* and *-15*. These results demonstrate that transit of the ϕ29 lysozyme across the cytoplasmic membrane of *E. coli* depends on the function of gene *14*.

In addition, we tested whether premature lysis of MC4100I^{Q}(pSB29-1) is inducible by addition of energy poisons, a hallmark of the lysis systems of *E. coli* phage lambda and *S. typhimurium* phage P22 (2, 18). *E. coli* MC4100I^{Q} carrying plasmids pSB29-1 and pSB29-2 was grown to an optical density at 600 nm of 0.2, at which time expression of genes *14* and *15* from plasmid pSB29-1 or expression of genes *sus14* and *-15* from plasmid pSB29-2 was induced. Twenty-five minutes later, cyanide was added to both cultures. Premature lysis was triggered immediately after addition of the energy poison to the gene *14* wild-type clone (not shown), whereas no lysis was observed with cells harboring plasmid pSB29-2, which carries the *sus14* allele. These experiments clearly showed that abolishing the membrane potential triggers protein 14, permitting release of the lysozyme to the periplasm, which in turn results in cell lysis.

Given that ϕ29-induced lysis in *B. subtilis* is only delayed in the absence of protein 14 (6), the question arises of the advantage of it in ϕ29 development. Cyanide-triggered premature lysis was strictly dependent on the presence of protein 14, exactly as described previously for lambda S (2). Since the gene *14* function seems to respond to the physiological state of the host, it might provide an environmentally responsive component in phage development. Thus, gene *14*, like its lambda analog *S*, might serve two functions: (i) proper scheduling of host cell lysis, and (ii) perhaps more importantly, provision of an intrinsic function to prevent phage entrapment in a host cell. Dissipation of the membrane potential due to environmental effects would instantaneously result in lysis and thus in release of phage progeny.

The lesion in the cytoplasmic membrane of *E. coli* induced by ϕ29 protein 14 is nonspecific. Next, we addressed the question of whether the lesion induced by protein 14 in the inner membrane of *E. coli* is nonspecific. We tested whether or not murein hydrolases unrelated to the ϕ29 lysozyme are released to the periplasm by protein 14. As shown in Fig. 3B, simultaneous expression of ϕ29 gene *14* and the lambda transglycosylase gene *R* (1) from plasmids pSB29-11 (*14+/15−*) and pJG16 (*S−*, *R+*, and *Rz−*), respectively, resulted in lysis after induction. In contrast, no lysis occurred when the *R* gene was expressed together with the *sus14* allele from plasmid pSB29-22 (not shown).

Similar results were obtained when phage T7 gene *3.5* encoding an amidase (11) was expressed from plasmid pLysS along with gene *14* from plasmid pSB29-11. Lysis was obtained 40 min after induction (Fig. 3B). Again, no lysis was observed when T7 gene *3.5* was expressed together with the *sus14* allele (not shown). When ϕ29 gene *14* was expressed along with T7 gene *3.5*, a delayed onset of lysis was observed when compared with the combination ϕ29 gene *14*/lambda gene *R* (Fig. 3B). This may be explained by the low expression rate of the T7 gene *3.5* from plasmid pLysS (26). These results indicate that protein 14 induces a nonspecific lesion in the inner membrane of *E. coli* through which murein hydrolases structurally and functionally unrelated to the ϕ29 lysozyme can pass.

The data presented strongly suggest that a dual lysis system involving a hole-forming protein and at least one murein hydrolase is not restricted to phages of gram-negative bacteria. Thus, our finding that ϕ29 protein 14 induces a nonspecific lesion unifies phage lysis of gram-positive and gram-negative bacteria in terms that the same strategy is elaborated. It also appears that hole-forming proteins have common structural traits regardless of their primary se-

quence. These similarities may serve for identification of hole-forming proteins as more sequence data become available for putative lysis genes of phages infecting gram-positive bacteria. In contrast to gram-negative bacteria, a nonspecific lesion in the membrane of gram-positive bacteria would result in release of cellular compounds. Thus, such hole-forming functions may have a potential application in dairy biotechnology as well as for utilization in conditional suicide systems designed for biological containment.

ACKNOWLEDGMENTS

We are grateful to K. Tedin and R. Young for comments on the manuscript and to M. Salas, R. K. Holmes, and F. W. Studier for providing phages, bacterial strains, and plasmids.

This work was funded by grant GZ. 49703 from the Austrian Bundesministerium für Wissenschaft und Forschung to W.L. and U.B., who was also supported by a grant from the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (P8309-BIO).

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