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Dual Translational Start Motif Evolutionarily Conserved in the Holin Gene of *Bacillus subtilis* Phage ϕ29

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Holins represent phage encoded lysis functions required for transit of the phage murein hydrolases to the periplasm. The Lambda *S*, phage 21 *S*, and P22 *13* holin genes contain a dual translational start motif, beginning with Met1–Lys2–X–Met3. In all cases both start codons at the 5′ end of the respective holin gene are utilized. The resulting polypeptides have opposing functions, with the longer product acting as an inhibitor of the shorter one. The 131-codon gene *14* of *Bacillus subtilis* phage ϕ29 encodes the holin function, whereas the downstream gene *15* codes for a lysozyme. ϕ29 Gene *14* begins with Met1–Lys2–Met3. Here, we present *in vitro* and *in vivo* evidence for the expression of two protein 14 species consisting of 129 and 131 amino acids, respectively. These data suggest that the lysis control mechanism based on two holin species, which has been shown to be operational in the temperate *Escherichia coli* phages Lambda and 21, and in the *Salmonella typhimurium* phage P22, is evolutionarily conserved in the lytic *B. subtilis* phage ϕ29.

INTRODUCTION

Large phage appear to employ, in general, at least two lysis functions, a holin and a murein-degrading enzyme (endolysin). Since all endolysins characterized to date are devoid of a signal sequence, they require the action of holins for their release to the murein, which seems to be achieved through the formation of a nonspecific lesion in the inner membrane (reviewed by Young, 1992). Holin functions have been attributed to the Lambda *S* gene (Young, 1992; Zagotta and Wilson, 1990), the P22 gene *13* (Renell and Poteete, 1985), and phage 21 gene *S* (Bonovich and Young, 1991). In addition, we have recently shown that the membrane-bound protein 14 of *B. subtilis* phage ϕ29 functions as a holin in *Escherichia coli* (Steiner *et al.*, 1993). In Lambda, P22, phage 21, and ϕ29 the gene arrangements of the corresponding holin and murein hydrolase functions are identical. The gene encoding the holin in all cases precedes the gene encoding the murein hydrolase (Fig. 1) and overlaps with at least the ribosome-binding sequence of the downstream murein hydrolase gene (Renell and Poteete, 1985; Bonovich and Young, 1991; Daniels *et al.*, 1983; Garvey *et al.*, 1986). Furthermore, there are sequence similarities around the start of the holin genes depicted in Fig. 1. Each gene is preceded by two consecutive ribosome-binding sequences and has two potential in-frame start codons both of which have been shown to be utilized in Lambda *S* (Bläsi *et al.*, 1989), as well as in P22 gene *13* (Nam *et al.*, 1990). Some evidence has been obtained for the usage of the two starts in phage 21 gene *S* (Bonovich and Young, 1991).

The Lambda *S* gene-encoded polypeptides S107 and S105 (Fig. 1) starting at Met codons 1 and 3 of *S*, respectively, have opposing functions in lysis. The S107 protein (Fig. 1) apparently acts as an inhibitor of S105, which has been termed lysis-effector (Bläsi *et al.*, 1990). The inhibitory action of the Lambda lysis-inhibitor S107 has been attributed to the interaction of its Lys2 residue with the negatively charged inner side of the energized inner membrane (Bläsi *et al.*, 1990; Steiner and Bläsi, 1993). This interaction could restrain lysis-effector molecules S105 from proper membrane assembly when they are organized in heterooligomers with S107 (Raab *et al.*, 1988; Bläsi *et al.*, 1990). A model has been put forward for assembly of the S-dependent lesion (Young, 1992): In the first stage, insertion and oligomerization of both S proteins in the plasma membrane may cause a steadily increasing leakage of protons, which at some point may lead to the dissipation of the membrane potential. As a result, the interaction of S107 with the inner membrane is relieved. According to the model, this would allow proper assembly of both proteins, i.e., formation of the lesion in the inner membrane. It has been suggested that this lysis control mechanism could serve proper scheduling of host cell lysis during lytic phage development (Raab *et al.*, 1988). In addition, the dual start motif (i.e., the lysis inhibitor S107) could serve to protect against readthrough at the late terminator t_r and may thus be important for establishment or maintenance of the lysogenic state (Nam *et al.*, 1990).

All features of the dual start motif, previously identified in holin genes of at least three lambdoid phage, appear to be present in ϕ29 gene *14* (Fig. 1). Here, we show that the molecular lysis control mechanism based on a lysis-effector and a lysis-inhibitor is conserved in ϕ29.

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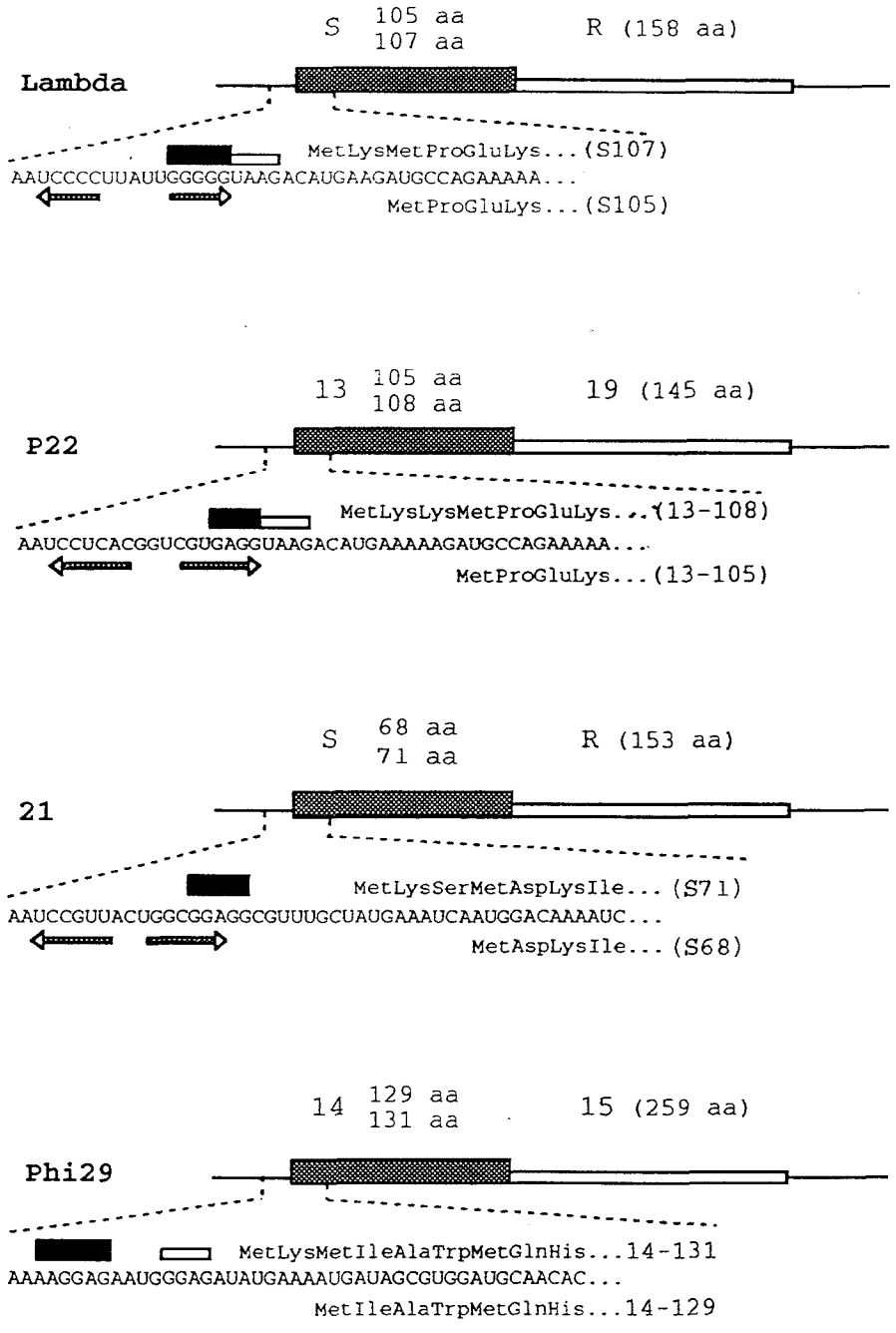

FIG. 1. Dual start motifs in holin genes. Partial maps of the lysis gene regions and the translational initiation region as well as the corresponding amino-terminal coding sequences of the holin genes (hatched bar) from the coliphages Lambda and 21, *Salmonella* phage P22, and *Bacillus* phage ϕ29 are shown. Inverted pairs of arrows indicate stem-loop structures demonstrated for Lambda *S* and P22 gene *13* (Bläsi *et al.*, 1989; Nam *et al.*, 1990) and inferred for the *S* gene of phage 21 (Bonovich and Young, 1991). The ribosome-binding sequences for the longer (black box) and shorter (open box) reading frames are indicated. These have been documented for Lambda *S* and P22 gene *13* and are inferred for the other holin genes. The number of amino acids (aa) for the longer and shorter product is given in each case above the corresponding holin gene. The downstream murein hydrolase genes are represented in either case by an open bar.

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids

E. coli strain MC4100I^{Q} (*araD* Δ(*argF-lacIOP-ZYA*) *U169 rpsL relA1 flbB5301 deoC1 ptsF25 rbsR* (F'*proAB lacI*^{Q} *Z*Δ*M15* Tn *10*)) and the *Bacillus subtilis* strain 110NA *su-* have been described (Steiner *et al.*, 1993). Phage ϕ29 was kindly provided by M. Salas, University of Madrid. Plasmids pKS^{-} (Stratagene Cloning Systems) and pAC9 (Bläsi *et al.*, 1990) have been described elsewhere.

Cells were routinely grown at 37° in LB broth (Miller, 1972) supplemented with the antibiotics as described in the text. Cell growth and lysis was monitored by measuring the optical density at 600 nm.

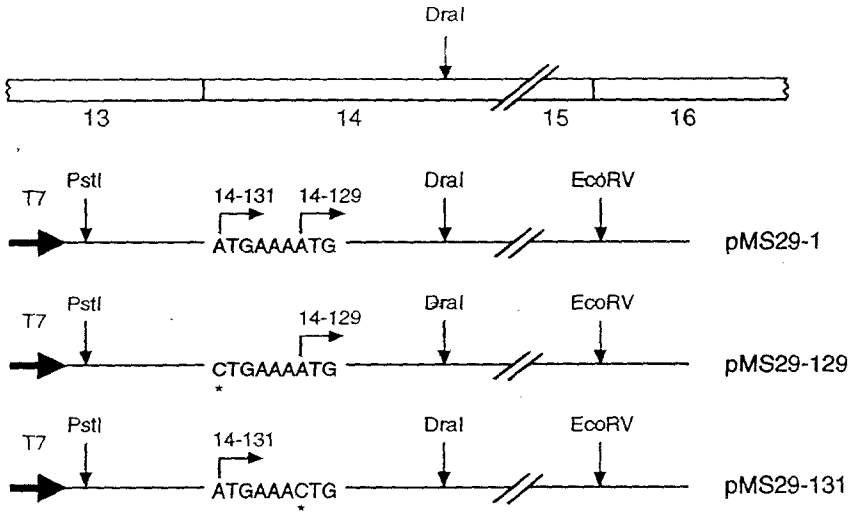

FIG. 2. Plasmids carrying the ϕ29 *14/15* region and the *14-131* and *14-129* alleles. The top section shows a partial map of the ϕ29 late genes (not drawn to scale). The tandem translational starts of gene *14* are shown by arrows. The stars below the DNA sequence mark the single base changes created by *in vitro* mutagenesis. Only relevant restriction sites are shown.

Construction of plasmids bearing ϕ29 genes *14* and *15*

The DNA region comprising the ϕ29 holin gene *14* and the lysozyme gene *15* was obtained from ϕ29 DNA by amplification using the polymerase chain reaction (Sambrook *et al.*, 1989) and the synthetic oligonucleotides A: 5′-CTTTTACTATCTGCAGCGCCC-3′ and B: 5′-GCGATATCAAAATCAACTTAATCTAATTGTTTGACC-3′. Oligonucleotide A anneals to base pair (bp) −38 to −57 upstream of the start codon of gene *14*. It was designed to create a *PstI* restriction site at position −44 relative to the A of the gene *14* AUG start codon 1 (see Fig. 1). Oligonucleotide B is complementary to the last eight codons of gene *15*. The 12 protruding bases at its 5′ end contain an *EcoRV* site. The resulting 1244-bp fragment was gel purified and subsequently digested with *PstI* and *EcoRV*. The *PstI*–*EcoRV* *14/15* containing fragment was inserted into the *PstI* and *EcoRV* sites of plasmid pKS$^-$. The resulting plasmid, pMS29−1 (Fig. 2), carries the ϕ29 genes *14/15* under transcriptional control of a T7 promoter. Plasmids pMS29-129 and pMS29-131 harboring the *14-129* allele (Met1 → Leu) and the *14-131* allele (Met3 → Leu), respectively, were constructed in the same way. Primer C (5′-ATAACTGCAGGTTGGAAAGCATAGAAAAGGAGAATGGGAGATCTGAAAATGATAGCG-3′: underlined bases indicate the *PstI* site and the A → C change in Met codon 1) was used to engineer a *PstI* site 43 bp upstream of the A of gene *14* codon Met3, and to convert Met codon 1 of gene *14* (*14-129* allele) to a Leu codon (Fig. 2). Primer D (5′-ATAACTGCAGGTTGGAAAGCATAGAAAAGGAGAATGGGAGATATGAAACTGATAGCG-3′: underlined bases indicate the *PstI* site and the A → C change in Met codon 3) was used to create a *PstI* site 37 bp upstream of the A of gene *14* Met codon 1 and to change Met3 to a Leu codon (*14-131* allele). The respective *14-129/15* and *14-131/15* cassettes were then cloned into pKS- resulting in plasmids pMS29-129 (*14-129/15*) and pMS29-131 (*14-131/15*) (Fig. 2).

The *14/15*, *14-129/15*, and *14-131/15* arrangements were isolated on *PstI*/*SalI* fragments from plasmids pMS29-1, pMS29-129, and pMS29-131, respectively, and inserted into the low copy number plasmid pAC9 (Bläsi *et al.*, 1990) linearized with *PstI* and *SalI*. In the resulting plasmids pAC29 (*14/15*), pAC29-129 (*14-129/15*), and pAC29-131 (*14-131*), the ϕ29 lysis genes are under transcriptional control of the *lac* promoter/operator.

In vitro transcription and toeprinting

For toeprinting (Hartz *et al.*, 1988), 5 μg of DNA of plasmids pMS29-1, pMS29-129, and pMS29-131, respectively, was digested with *DraI* (see Fig. 2) and then transcribed *in vitro* with T7 RNA polymerase (Sambrook *et al.*, 1989) and the Riboprobe Gemini System II as recommended by the manufacturer. A 0.5-μg amount of *DraI*-restricted pMS29-1 was used as template for marker RNA synthesis in the presence of [α-^{32}P]UTP. The different gene *14*-specific mRNAs were then purified on an 8% polyacrylamide–urea gel, suspended in 30 μl of 0.1 m*M* EDTA, and used for toeprinting. A 10-pmol aliquot of the synthetic oligonucleotide primer 5′-CCATAAGGAATGTTAACCAG-3′, complementary to nucleotides +60 to +79 of gene *14*, was labeled with 20 pmol of [γ-^{32}P]ATP. Primer labeling, annealing of the labeled primer to the gene *14* mRNA species, primer extension, toeprinting with *E. coli* 30S ribosomes, and dideoxy sequencing of gene *14* and the *14-129* and *14-131* alleles, respectively, were carried out exactly as described before for the Lambda *S* gene (Bläsi *et al.*, 1989).

In vitro translation

E. coli S-30 extracts were prepared from strain MS59 (Δ*lac*, *supE*, *supF*) using the methodology of Zubay

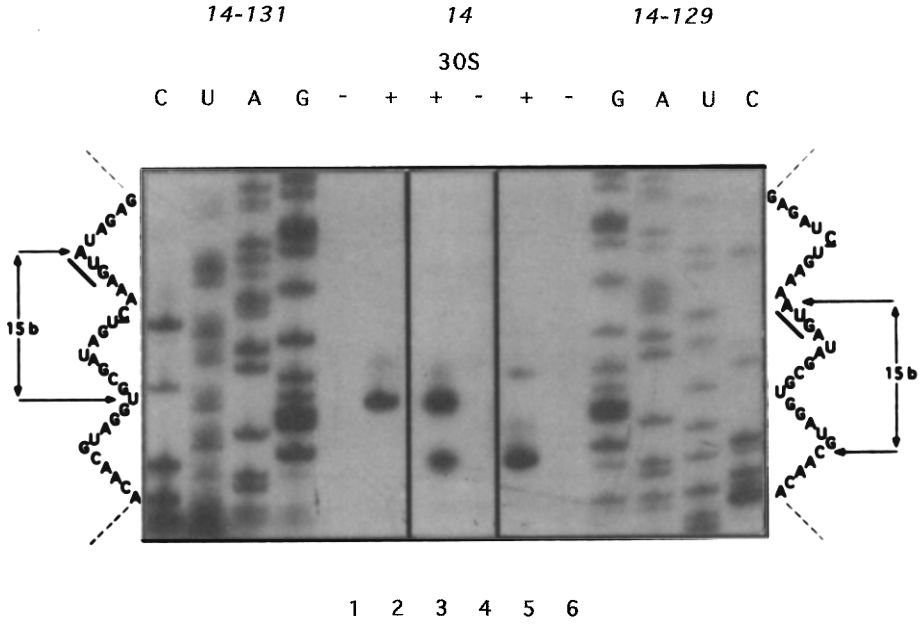

FIG. 3. Toeprint analysis of gene *14* and the *14-129* and *14-131* alleles. Toeprinting with *E. coli* 30S subunits was performed as described under Materials and Methods. The mRNA sequence of the 5′ flanking and initial coding sequences of the *14-131* and *14-129* alleles are given at the left and at the right, respectively. Start codons 1 or 3 are marked with a bar. The A → C changes in start codon 3 (*14-131* allele) and in start codon 1 (*14-129* allele), respectively, are underlined. The (−) sign indicates primer extension reactions with *14-131* mRNA (lane 1), *14* mRNA (lane 4), or with *14-129* mRNA (lane 6) in the presence of $tRNA^{fMet}$ but without 30S ribosomes added. Primer extension inhibition analysis in the presence of $tRNA^{fMet}$ and 30S ribosomes are marked with a (+) sign. Lane 2, Toeprint analysis of the *14-131* allele. Lane 3, Toeprint analysis of gene *14*. Lane 5, Toeprint analysis of the *14-129* allele.

(1980). *B. subtilis* S-30 extracts were prepared from *B. subtilis* strain 110NA according to a protocol of Farwell and Rabinowitz (1991). Radioactive labeling was performed with Trans[^{35}S]Label from ICN Biomedicals, Irvine. *In vitro* translated proteins were separated on 40-cm SDS−polyacrylamide gels as described before (Bläsi *et al.*, 1990). For *in vitro* translation, transcripts of genes *14* and *15* and of the *14* mutant alleles along with gene *15* were obtained using T7 RNA polymerase after digestion of the plasmids of the pMS series (Fig. 2) with *Eco*RV.

RESULTS AND DISCUSSION

Toeprinting of gene *14* reveals dual translational starts

Recent *in vivo* expression experiments with ϕ29 gene *14* in *E. coli* suggested a doublet at the position of protein 14 in SDS−polyacrylamide gels (Steiner *et al.*, 1993). To test directly whether both initiation codons at the 5′ end of gene *14* are used for translation initiation, primer extension inhibition analysis (toeprinting) was performed on gene *14* mRNA comprising the first 51 codons. Toeprinting has been used to reveal the site of 30S ribosomal subunits bound *in vitro* in ternary complexes (Hartz *et al.*, 1989). Briefly, a toeprint signal is generated when elongation of a downstream-primed cDNA by reverse transcriptase is stopped at the 3′ edge of the ribosomal subunit. The toeprint signal occurs usually 15 nt downstream of the adenosine of the initiation codon. Toeprinting on gene *14* mRNA using *E. coli* 30S ribosomes revealed toeprint signals at position +15 relative to the As of both start codons Met1 and Met3, respectively (Fig. 3, lane 3). According to the toeprint signals, initiation events appear to occur at least twofold more frequently at Met1 than at Met3. To test whether the respective signals obtained for both Met codons are specific, toeprint analysis was also performed on gene *14* alleles in which either Met codon 1 (*14-129* allele) or 3 (*14-131* allele) was converted to a Leucine. Toeprint analysis on *14-129* mRNA (Fig. 3, lane 5) resulted in only a +15 signal relative to the A of the Met3 codon, whereas the corollary toeprint analysis on *14-131* mRNA confirmed the specificity of the signal for Met1 (Fig. 3, lane 2). These experiments strongly suggest that both start codons, Met1 and Met3, are used as translational starts in *E. coli.* There are two putative ribosome-binding elements upstream of the gene *14* coding region which could serve the two start codons (see Fig. 1), Met1 and Met3. Using the method of Freier *et al.* (1986), the subsequence 5′-AAGGAG-3′ located at position −16 to −11 relative to the A of start codon Met1 (Fig. 1) has a calculated free energy of binding to *E. coli* 16S rRNA of −6.1 kcal/mol. Since the spacing between this putative element and the first nucleotide of Met codon 1 is 10 nucleotides, considered to be optimal (Hartz *et al.*, 1991), we suggest that it serves for starts at Met1. Likewise, we consider the subsequence 5′-GAGG-3′ (Fig. 1), which has a less extended complementarity to the 16S rRNA ($\blacktriangle G$ = −5.2 kcal/mol) and a

distance of 8 nucleotides relative to the A of Met codon 3, to direct translational starts at Met3. The partitioning of ternary complex formation between the two start codons of gene *14* (Fig. 3, lane 3) could thus be explained by the relative strength of the corresponding ribosome-binding sequences.

In vitro translation of gene *14* with *E. coli* and *B. subtilis* S-30 extracts results in the production of two protein 14 species.

We next asked whether expression of gene *14* results in the production of two protein species in *E. coli* and *B. subtilis*. First, ϕ29 gene *14* and *14-129* and *14-131* alleles were transcribed and translated *in vitro* using *E. coli* S-30 extracts. As shown in Fig. 4, lane 1, two protein 14 species were resolved after expression of gene *14* employing *E. coli* extracts. The ratio of the translation products was 2:1 in favor of the longer polypeptide, 14-131. Translation of the pMS29-129 (Met1 → Leu) transcript and the pMS29-131 (Met3 → Leu) transcript with *E. coli* extracts revealed only signals for protein 14 species consisting of 129 and 131 amino acids, respectively, as expected (Fig. 4, lanes 2 and 3). Basically the same results were obtained when *B. subtilis* S-30 extracts were programmed with the pMS29-1 transcript comprising gene *14* (Fig. 4, lane 4). Two protein 14-specific bands were resolved. It should be noted that the ratio of proteins 14-131 to 14-129 obtained with *B. subtilis* extracts was approximately 2:1, as with *E. coli* extracts. Moreover, the *in vitro* translation data confirmed the partitioning of translation initiation events at Met1 and Met3 as revealed by the toeprint experiments (see Fig. 3, lane 3).

Gene *14* encodes a lysis-effector and a lysis-inhibitor

Protein 14 has been shown recently to induce a non-specific lesion in the *E. coli* inner membrane (Steiner *et al.*, 1993). To assess whether the two protein 14 species function as lysis-inhibitor and lysis-effector, respectively,

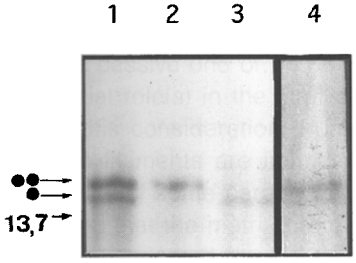

FIG. 4. *In vitro* translation of ϕ29 gene *14* and the *14-129* and *14-131* alleles using *E. coli* and *B. subtilis* S30 extracts. Only the relevant section of the SDS–polyacrylamide gel is shown. The *E. coli* S-30 *in vitro* translation products of transcripts carrying the *14*, *14-131*, and *14-129* alleles are shown in lanes 1, 2, and 3, respectively. The positions of protein 14-131 (●●) and 14-129 (●) species as well as that of a molecular weight marker are indicated at the left by arrows. Lane 4, gene *14*-specific translation products obtained with *B. subtilis* S-30 extracts.

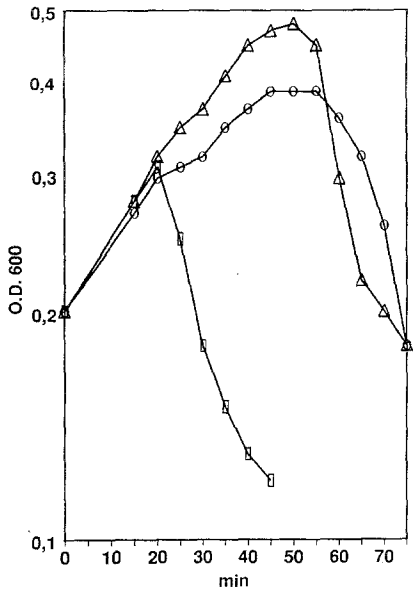

FIG. 5. Expression of the gene *14* and the *14-129* and *14-131* alleles in *E. coli*. *E. coli* strain MC4100I^{Q} (see text) harboring plasmids pAC29 (○, encodes 14-129 and 14-131 proteins and protein 15), pAC29-129 (□, encodes the 14-129 species and protein 15), and pAC29-131 (△, encodes the 14-131 species and protein 15), respectively, was grown at 37° in LB broth (Miller, 1972) supplemented with 50 μg/ml kanamycin. At time 0, expression of the cloned lysis genes was induced by addition of 5 m*M* IPTG (final concentration).

the ϕ29 gene *14* and the *14-131* and *14-129* alleles were cloned along with ϕ129 gene *15* (lysozyme) under transcriptional control of the *lac* promoter/operator in a low copy number *E. coli* plasmid. The plasmids pAC29 (genes *14/15*), pAC29-131 (*14-131*: Met3 → Leu; gene *15*), and pAC29-129 (*14-129*: Met1 → Leu; gene *15*), respectively, were introduced into the LacI-overproducing *E. coli* strain MC4100I^{Q}. Expression of the *14/15* cassette from plasmid pAC29 resulted in cessation of growth 44 min after induction with a decrease in cell density, i.e., lysis, 55 min after induction (Fig. 5). Induction of the *14-129* (Met1 → Leu) allele together with gene *15* in transformants harboring plasmid pAC29-129 was followed by rapid lysis 20 min after induction (Fig. 5). The *14-131* (Met3 → Leu) allele in plasmid pAC29-131 resulted in lysis onset 50 min after induction (Fig. 5). These experiments clearly show that the shorter product 14-129 has a higher lethal potential than the longer product 14-131, just as previously shown for the analogous Lambda S species (Raab *et al.*, 1988; Bläsi *et al.*, 1989). In addition, the delay in lysis observed with the wild-type gene *14* relative to the *14-129* (Met1 → Leu) allele can be interpreted as showing that the longer product affects the lethal potential of the shorter product.

In contrast to the Lambda lysis-inhibitor S107 (Bläsi *et al.*, 1989) and the P22 lysis-inhibitor 13-108 (Nam *et al.*, 1990), the longer species 14-131 is produced in both *E. coli* and *B. subtilis* 30S extracts at a higher level than the shorter product 14-129. Furthermore, protein 14-131 shows a lethal potential, i.e., is apparently forming a le-

sion in the inner membrane when produced from plasmid pAC29-131 in the absence of the 14-129 protein (Fig. 5). We have recently shown that moderate expression of the Lambda lysis-inhibitor protein S107 together with the R transglycosylase does not result in cell lysis, whereas overproduction of S107 results in lysis in the presence of R (Steiner and Bläsi, 1993). A possible explanation for this finding may be that insertion of a large number of S107 molecules into the inner membrane at some point leads to dissipation of the membrane potential. As a consequence, an S107-dependent lesion(s) forms in the cytoplasmic membrane. In fact, it has been shown previously that KCN-triggered collapse of the membrane potential results in formation of a lesion in the inner membrane by S107 alone (Bläsi *et al.,* 1990). Analogously, the lethal capacity of 14-131, when expressed from pAC29-131, may be explained by its high-level production directed by the "strong" ribosome-binding sequence 5′-AAGAGG-3′ (Fig. 1). The high lethal capacity of protein 14-129 (lysis onset occurring after 20 min) could, in contrast to Lambda *S*, require the observed higher production of protein 14-131 (Figs. 3 and 4) relative to protein 14-129 for proper scheduling of host cell lysis. Although there are some differences among analogous systems found in lambdoid phage, the conservation of the dual start motif in the holin gene of the lytic *B. subtilis* phage ϕ29, unrelated to the temperate *Enterobacteriaceae* phages Lambda, P22 or 21, seems to underscore the importance of this intricate lysis control mechanism in phage development.

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