JOURNAL OF BACTERIOLOGY, Feb. 1995, p. 723–732 Vol. 177, No. 3
0021-9193/95/$04.00+0

Primary Structure and Functional Analysis of the Lysis Genes of *Lactobacillus gasseri* Bacteriophage ϕadh

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Received 28 June 1994/Accepted 19 November 1994

The lysis genes of the *Lactobacillus gasseri* bacteriophage ϕadh were isolated by complementation of a lambda *S*am mutation in *Escherichia coli*. Nucleotide sequencing of a 1,735-bp DNA fragment revealed two adjacent coding regions of 342 bp (*hol*) and 951 bp (*lys*) in the same reading frame which appear to belong to a common transcriptional unit. Proteins corresponding to the predicted gene products, holin (12.9 kDa) and lysin (34.7 kDa), were identified by in vitro and in vivo expression of the cloned genes. The ϕadh holin is a membrane-bound protein with structural similarity to lysis proteins of other phage, known to be required for the transit of murein hydrolases through the cytoplasmic membrane. The ϕadh lysin shows homology with mureinolytic enzymes encoded by the *Lactobacillus bulgaricus* phage mv4, the *Streptococcus pneumoniae* phage Cp-1, Cp-7, and Cp-9, and the *Lactococcus lactis* phage ϕLC3. Significant homology with the N termini of known muramidases suggests that ϕadh lysin acts by a similar catalytic mechanism. In *E. coli*, the ϕadh lysin seems to be associated with the total membrane fraction, from which it can be extracted with lauryl sarcosinate. Either one of the ϕadh lysis proteins provoked lysis of *E. coli* when expressed along with holins or lysins of phage lambda or *Bacillus subtilis* phage ϕ29. Concomitant expression of the combined holin and lysin functions of ϕadh in *E. coli*, however, did not result in efficient cell lysis.

Large *Escherichia coli* phage in general appear to encode at least two lysis functions, a murein hydrolase, required for destruction of the peptidoglycan, and a protein termed holin which permits access of the lytic enzyme to the periplasm (for a review, see reference 78). In the case of bacteriophage lambda, oligomerization of the holin (protein S) in the inner membrane of *E. coli* apparently leads to formation of a nonspecific lesion through which the lambda transglycosylase is released to the periplasm at the end of the vegetative cycle (80). The expression of the *S* gene and thus the kinetics of formation of the S-dependent hole in the inner membrane is tightly controlled at the transcriptional (35) and at the translational level (7, 53), as well as posttranslationally by virtue of two *S*-encoded polypeptides with opposing functions (6, 70). Holin functions have also been attributed to the products of P22 gene *13* (58), to phage 21 gene *S* (9), and recently to protein 14 of the *Bacillus subtilis* phage ϕ29 (70), the first holin identified from a phage of gram-positive bacteria. Large phage therefore appear to pursue an evolutionarily conserved lysis pathway.

In phage lambda, P22, 21, and ϕ29, the genes encoding the corresponding holins and murein hydrolases are arranged identically. The holin gene in all cases precedes the gene encoding the murein hydrolase and overlaps at least with its ribosome-binding site (9, 13, 30, 58). With the exception of the products of the lambda *S* and P22 *13* genes, which are nearly identical (58), the known holins show no homology with each other (78). Since lambda S protein and ϕ29 protein 14 have been shown to function in *Saccharomyces cerevisiae* and *E. coli*, respectively, formation of nonspecific membrane lesions by holins may require nothing more than a lipid bilayer (28, 70).

Several murein hydrolases of phage infecting *Streptococcus pneumoniae* have been analyzed. These include the muramidases of the phage Cp-1, Cp-7, and Cp-9 (24, 26) and the amidases of phage EJ-1 (16) and HB-3 (60). The N-terminal domains of the Cp-1, Cp-7, and Cp-9 muramidases are closely related to each other. In addition, the C termini of the Cp-1 and Cp-9 enzymes are homologous to the sequence repeats found in the C-terminal cholate-binding domain of the major pneumococcal autolysin, the LYTA amidase (25). The amidases of phage EJ-1 and HB-3 are also very similar to the host-encoded LYTA enzyme at both their N- and C-terminal ends (16, 59). Another murein hydrolase with sequence homology to the LYTA amidase has been isolated from the *Lactococcus lactis* phage ϕUS3 (51). The peptidoglycan-degrading enzymes encoded by the *B. subtilis* phage PZA and ϕ29 are considered to act as muramidases because they share homology with the T4 lysozyme and P22 protein 19 (62). A lysozyme-like activity has also been proposed for the *Lactococcus lactis* phage ϕvML3 (67). All murein hydrolases thus far described for phage of gram-positive bacteria have in common the lack of an apparent signal sequence for transit across the cytoplasmic membrane (78).

The recent demonstration that phage ϕ29 protein 14 is operational in *E. coli* and that it can substitute for lambda protein S in cell lysis prompted us to attempt the isolation of a holin function of a *Lactobacillus* phage by complementation of a lambda *S*am mutation in *E. coli*. Homologous holin functions may be a valuable future tool for the release of nonsecretory proteins produced by means of recombinant DNA technology in food-grade lactobacilli. Here, we report the characterization of the lysis functions of the temperate *Lactobacillus gasseri* phage ϕadh, which, by phenotypic criteria, belongs to the *Siphoviridae* family. The genome of ϕadh consists of double-stranded DNA of 43 kb with cohesive ends (56). Phage ϕadh was shown to mediate high-frequency transduction of plasmids carrying particular fragments of ϕadh DNA (55), and the attachment sites involved in integration of ϕadh into the chromosome of the host have recently been determined (54).

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TABLE 1. Strains, phage, and plasmids used

Strain, phage, or plasmid	Genotype or description	Source (reference) or reference
Strains		
E. coli K-12		
CAI^{Q1}	Δ(*lac pro*) *thi cap cya strA recA* F′ (*lacI*^{q1} *lac*$^{+}$ *pro*$^{+}$)	U. Rüther (33)
Y1088	*e14* (*mcrA*) Δ(*lac*)U169 *supE supF hsdR metB trpR tonA21 proC*::Tn*5* [pMC9]	Stratagene (79)
MC4100	*araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Stratagene (69)
MC4100I^{Q}	MC4100 F′ (*proAB lacI*^{q} *Z*ΔM15 Tn*10*)	70
L. gasseri ADH	Human isolate, host for ϕadh	T. R. Klaenhammer (56)
Phage		
E. coli		
Lambda gt11	*c*I857, *S*100, p_{lac} *lacZ*, insertion vector	Stratagene (79)
Lambda 111	*c*I857, *S*am7	Laboratory stock
Lambda CE6	*c*I857, *S*am7, *int*::(T7 gene *1*)	F. W. Studier (72)
L. gasseri ϕadh	Temperate phage, classified within the *Siphoviridae* family	T. R. Klaenhammer (56)
Plasmids		
pUC19	Ap^{r}, p_{lac} *lacZ′*, ColE1 origin	Boehringer (76)
pBluescriptSK+	Ap^{r}, p_{lac} *lacZ′*, T7p, T3p, ColE1 origin, f1 origin	Stratagene
pK184/pK194	Km^{r}, p_{lac} *lacZ′*, p15a origin	M. G. Jobling (40)
pDR540	Ap^{r}, p_{tac} *galK*, ColE1 origin	Pharmacia
pUC19dtac	pUC19::91-bp *Bam*HI-*Hin*dIII fragment from pDR540	This work
pLS130	Ap^{r}, lambda p_{L} *S*$^{-}$(Met-1,3→Leu) *R* R_{Z}, ColE1 origin	7
pKS^{-}R^{+}	pK184::1,478-bp *Eco*RI-*Hin*dIII fragment from pLS130	This work
pSB29-2	pK194 derivative, with ϕ29 allele sus*14* and gene *15* under p_{lac}	70
pSB29-11	pK194 derivative, with ϕ29 gene *14* under p_{lac}	70
pLysS	pACYC184 derivative, with T7 gene *3.5*	F. W. Studier (71)
pUC19hol	pUC19::432-bp PCR product containing *hol* of ϕadh	This work
pUC19lys	pUC19::1,011-bp PCR product containing *lys* of ϕadh	This work
pUC19holys	pUC19::1,397-bp PCR product containing *hol* and *lys* of ϕadh	This work
pSKhol	pBluescriptSK+::432-bp PCR product containing *hol* of ϕadh	This work
pSKlys	pBluescriptSK+::1,011-bp PCR product containing *lys* of ϕadh	This work
pSKholys	pBluescriptSK+::1,397-bp PCR product containing *hol* and *lys* of ϕadh	This work
pK194hol	pK194::432-bp PCR product containing *hol* of ϕadh	This work
pK194lys	pK194::1,011-bp PCR product containing *lys* of ϕadh	This work

MATERIALS AND METHODS

Bacterial strains, phage, plasmids, and growth conditions. The bacteria, phage, and plasmids used in this study are listed in Table 1. Bacteriophage lambda was plated on *E. coli*, and strain MC4100I^{Q} was lysogenized with lambda 111 according to standard procedures (63). Media used were Luria-Bertani (LB) medium or M9 minimal medium for *E. coli* (48) and MRS medium for *L. gasseri* (14). Antibiotics were added to the media as required: ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, and tetracycline at 30 μg/ml.

Recombinant DNA techniques. Standard techniques were used for the preparation and analysis of plasmid DNA (63). Restriction endonucleases and nucleic acid-modifying enzymes were used as recommended by the manufacturers. DNA fragments were transferred to Hybond-N+ membranes (Amersham), probes were labelled with fluorescein-11-dUTP, and hybridization was performed as specified by the supplier (Amersham) of the enhanced chemiluminescence kit used. *E. coli* was transformed by electroporation (17), using a Gene Pulser (Bio-Rad). All primers for PCR and nucleotide sequencing were synthesized on an Applied Biosystems model 392 DNA/RNA synthesizer.

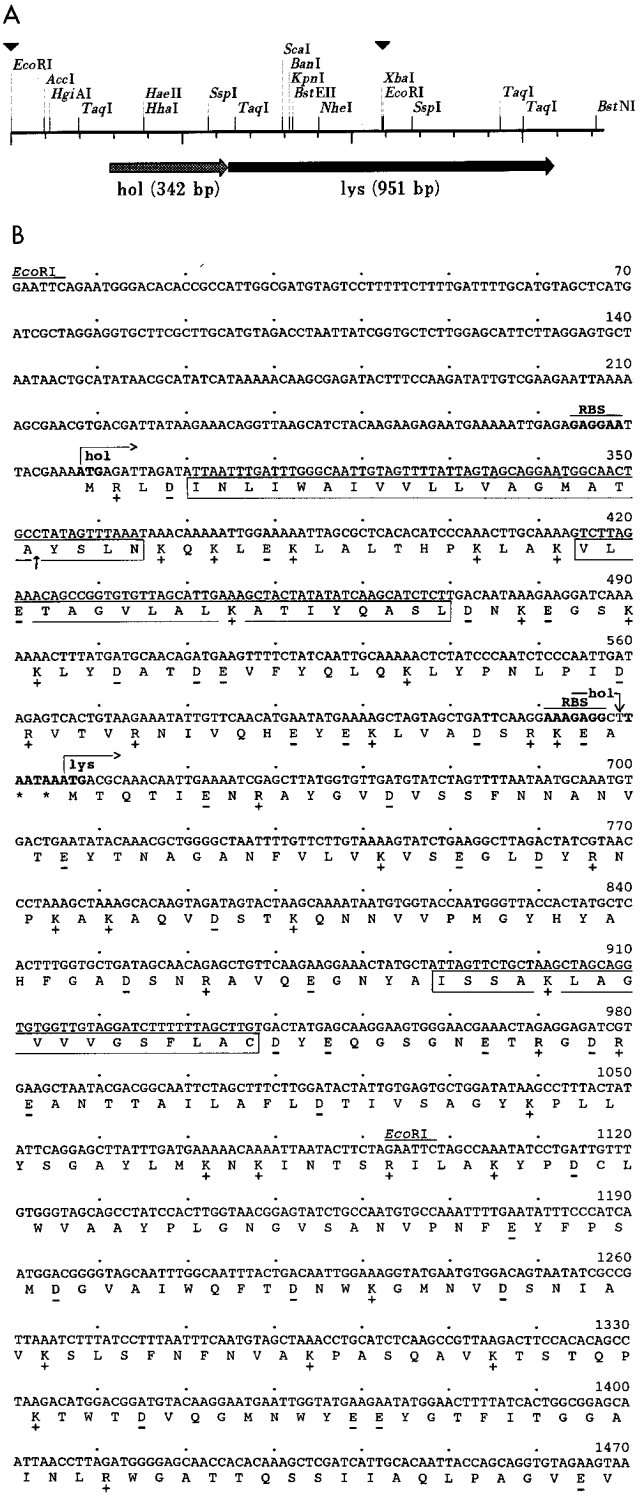
Cloning of ϕadh lysis functions. Phage ϕadh was propagated on *L. gasseri* ADH, and phage DNA was isolated as described by Raya et al. (56). After digestion of purified ϕadh DNA with *Eco*RI, at least 12 distinct fragments were detected by agarose gel electrophoresis. These fragments (50 ng) were ligated with lambda gt11 DNA that had been cut with *Eco*RI and treated with calf intestinal alkaline phosphatase (63). Ligation products were packaged in vitro in a total volume of 550 μl, using a GigapackII packaging extract (Stratagene) as recommended by the supplier. The packaged mixture plated with efficiencies of 1.1×10^{4} plaques per ml on strain MC4100 and 1.9×10^{6} plaques per ml on strain Y1088 (*supE supF*). From the lawn of strain MC4100, 10 single plaques were picked, and phage were eluted in volumes of 0.1 ml of SM buffer (63). Of these samples, 2.5 μl was used to perform PCR (in a Perkin-Elmer Cetus thermal cycler) in 25-μl reaction volumes with two oligonucleotide primers (5′-TGGC GACGACTCCTGGAGCCCG-3′ and 5′-TGACACCAGACCAACTGGTAAT GG-3′) hybridizing to the upstream and downstream regions of the unique *Eco*RI site in lambda gt11 DNA (49). Reaction mixtures were composed as recommended by the supplier (Perkin-Elmer) of the UlTma DNA polymerase used. After initial heating at 95°C for 4 min, a total of 30 cycles was carried out with denaturation at 95°C for 1 min, primer annealing for 1 min, and extension at 72°C for 1.75 min. The annealing temperature was successively lowered from 63°C (for the first 2 cycles) to 61°C (4 cycles), 59°C (5 cycles), 57°C (7 cycles), and 55°C (12 cycles). PCR products were digested with *Eco*RI and inserted into the unique *Eco*RI site of the vector pUC19dtac. To screen for recombinant plasmids, the *lac* repressor-overproducing strain CAI^{Q1} was transformed with the ligation mixture.

Construction of plasmids. The vector pUC19dtac is a derivative of pUC19 in which the 30-bp *Bam*HI-*Hin*dIII fragment from the polylinker region was replaced by the 91-bp *Bam*HI-*Hin*dIII fragment of plasmid pDR540, which carries the *tac* promoter.

To produce DNA fragments containing the isolated ϕadh genes *hol*, *lys*, and both *hol-lys*, the oligonucleotides listed below were used as primers for PCR in the presence of ϕadh DNA as a template. The sequences of the primers were identical or complementary to the upstream or downstream sequences of the regions to be amplified, with the exception of the underlined mismatches. Because of these mismatches, *Bam*HI and *Hin*dIII sites (shown in italics) were introduced in the sequences of the sense and antisense primers, respectively. The primers were 5′-AAAGCGAACGTG*A*A*G*C*TT*ATAAGAAACAGGTTAA GC-3′ (primer 1a, positions 209 to 241 in Fig. 1B), 5′-TCAACACCATAAGCT *G*G*ATCC*TCAATTGTTTGCG-3′ (primer 1b, positions 640 to 673), 5′-ATAT GAAAAGCTAG*A*A*GCTT*ATTCAAGGAAAGAGGC-3′ (primer 2a, positions 593 to 628), and 5′-TAGCTAAGCTCACGTAG*G*G*A*T*CC*TTAGCTTGG-3′ (primer 2b, positions 1609 to 1640). Using UlTma DNA polymerase (Perkin-Elmer), which has an inherent proofreading activity, amplification products were generated with primers 1a-1b, 2a-2b, and 1a-2b in separate reactions under standard PCR conditions (63). After digestion with *Hin*dIII and *Bam*HI, the resulting fragments of 432 bp (containing *hol*), 1,011 bp (containing *lys*), and 1,397 bp (containing *hol-lys*) each were cloned into *Hin*dIII-*Bam*HI-cut pUC19 (giving rise to pUC19hol, pUC19lys, and pUC19holys) and *Hin*dIII-*Bam*HI-cut pBluescriptSK+ (giving rise to pSKhol, pSKlys, and pSKholys). The 432- and 1,011-bp fragments were also inserted into *Hin*dIII-*Bam*HI-cut pK194 (giving rise to pK194hol and pK194lys).

Plasmid pKS^{-}R^{+} was constructed by ligation of the 1,478-bp *Eco*RI-*Hin*dIII fragment from pLS130, carrying the lambda *S*$^{-}$(Met-1,3→Leu) allele together with *R* and R_{Z} under transcriptional control of the lambda p_{L} promoter (7), into *Eco*RI-*Hin*dIII-digested pK184.

DNA sequencing and computer analyses. Automated DNA sequencing with an Applied Biosystems model 373A sequencer was performed on both strands of the analyzed DNA segment (Fig. 1). The sequence initially determined from the



1,093-bp *Eco*RI inserts of two individually obtained pUC19dtac derivatives (see Results) was verified with φadh DNA as the template. The sequence downstream of the *Eco*RI site at position 1093 (Fig. 1B) was directly established by using φadh DNA. Synthetic primers were designed from the sequence of the pUC19dtac vector and from already sequenced regions of φadh DNA. Database comparisons, alignments, and further analyses of nucleic acid and amino acid sequences were performed with the Microgenie (Beckman), Husar (Geniusnet), and PC/Gene (Intelligenetics) programs.

In vitro and in vivo gene expression. For in vitro synthesis of ^{35}S-labelled proteins, plasmid DNA was isolated by the Qiagen purification system (Diagen), and 0.5 μg was used as the template in a coupled *E. coli* transcription-translation system (United States Biochemicals). From the samples, aliquots (1/5 volume) were analyzed electrophoretically as described by Laemmli (45). Plasmid-directed synthesis of ^{35}S-labelled proteins by use of lambda CE6-encoded T7 RNA polymerase was carried out in 5-ml cultures in the presence of 100 μCi of L-[^{35}S]methionine (1,000 Ci/mmol; Amersham) as described previously (6). To analyze unlabelled proteins of cells containing cloned genes under p_{lac} control, the transformants were grown in LB medium at 37°C. At an optical density at 600 nm of 1, the cultures were diluted 40-fold with prewarmed medium containing 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and further incubated for 4 h.

Cell fractionation. The procedures of Filip et al. (21) and Ito et al. (39) were combined in the following protocol. Cells harvested from liquid culture (5 to 40 ml) were washed with 30 mM Tris hydrochloride (pH 8.1), resuspended in 0.4 ml of cold 20% (wt/vol) sucrose–30 mM Tris hydrochloride (pH 8.1), and treated with 1/10 volume of lysozyme (1 mg/ml) in 0.1 M EDTA (pH 7.3) for 30 min at 0°C. The spheroplasts were disrupted by four 15-s bursts of a Bandelin Sonoplus HD 60 sonicator and then diluted with an equal volume of 3 mM EDTA (pH 7.3). After removal of debris and unbroken cells by centrifugation at 2,000 × *g* for 10 min, an aliquot (1/4 volume) of the extract was subjected to ultracentrifugation in a Beckman 70.1 Ti rotor for 2 h at 60,000 rpm and 4°C, and the resulting supernatant was used as the cytoplasmic fraction. The total membrane pellet was washed twice with 30 mM Tris hydrochloride (pH 8.1), carefully suspended in 20 μl of 0.5% sodium lauryl sarcosinate (Sarkosyl NL-97; Serva), and shaken for 1 h at room temperature. The sample was centrifuged as before, and the supernatant was taken as the lauryl sarcosinate-soluble membrane fraction. The remainder of the extract obtained after low-speed centrifugation of the disrupted spheroplasts was layered onto 15% sucrose–3 mM EDTA (pH 7.3) (3.2 to 3.4 ml), with a cushion of 1 ml of 70% sucrose–3 mM EDTA (pH 7.3) at the bottom, and centrifuged in a Beckman SW50.1 rotor for 1 h at 50,000 rpm and 4°C. The total membrane fraction subsequently collected from the 15 to 70% interphase was layered onto 53% sucrose–3 mM EDTA (pH 7.3) (3 to 3.5 ml), with a cushion of 1 ml of 70% sucrose–3 mM EDTA (pH 7.3) at the bottom, and centrifuged in the SW50.1 rotor for 4 h at 50,000 rpm and 4°C. The bands subsequently collected from above the 53 and 70% sucrose layers were used as the cytoplasmic membrane and outer membrane fractions, respectively. They were diluted about 10-fold with 10 mM $MgCl_2$, and the membranes were pelleted by centrifugation in the SW50.1 rotor for 1 h at 50,000 rpm and 4°C. The resulting sediments were dissolved in 25 to 50 μl of sample buffer (45). Of the subcellular fractions obtained, 2 μl (in the case of radioactively labelled proteins) or 5 μl (in the case of unlabelled proteins) was analyzed electrophoretically (45).

Nucleotide sequence accession number. The GenBank accession number of the DNA sequence reported in this paper is X78410.

RESULTS

Identification of φadh lysis functions by complementation. To identify genes of phage φadh which are involved in host cell lysis, a library of *Eco*RI fragments of purified φadh DNA was established in lambda gt11 carrying an amber mutation in gene *S* (*S*100). Lambda gt11 is unable to lyse nonsuppressing *E. coli* hosts, since a functional gene *S* product is essential for the transit of lambda transglycosylase to the murein (57). Thus,

FIG. 1. Physical map and nucleotide sequence of the analyzed segment of φadh DNA. (A) Restriction and genetic map. The extent and direction of the *hol* and *lys* coding regions is given by arrows. Some pertinent restriction sites are shown. Arrowheads point to the ends of the initially cloned 1,093-bp *Eco*RI fragment. (B) Nucleotide sequence of the noncoding strand of the *hol-lys* region of φadh DNA. Numbering starts at the first residue of the left *Eco*RI site in panel A. Angled arrows indicate the 5′ and 3′ endpoints of the *hol* and *lys* coding regions. The deduced amino acid sequences of holin and lysin are shown below the DNA sequence. Putative transmembrane segments in both proteins are shown by boxes. A potential signal sequence cleavage site after residue 22 of the *hol* product is indicated by an upward arrow. Charged amino acid residues are marked by + and − signs below the protein sequences. Possible ribosome-binding sites (RBS) preceding the *hol* and *lys* genes and an inverted repeat (IR) capable of forming a stem-loop structure (positions 1691 to 1726) are indicated.

after in vitro packaging of the ϕadh library and subsequent infection of *E. coli* MC4100, plaque formation depended on complementation of the defective lambda *S* allele by analogous functions of phage ϕadh. The ratio of plaques obtained on strain MC4100 and on a *supE supF* reference strain (Y1088) was about 1:170. Inserts of ϕadh DNA contained in 10 recombinant plaques were amplified by PCR. Seven of the resulting products, which had the same size of approximately 1.1 kb, were cloned under transcriptional control of the tandem *lactac* promoter of plasmid pUC19dtac. Almost 50% of the resulting recombinants showed growth retardation after addition of IPTG to liquid cultures. All plasmids isolated from such clones yielded the same fragment pattern after restriction with *Taq*I, *Kpn*I, and *Eco*RI, suggesting that the growth retardation phenotype was orientation specific. The inserts of ϕadh DNA contained in two of these plasmids were sequenced.

One of them (named pUC19-3) was used as a probe to locate the cloned DNA region on the physical *Bgl*II restriction map of the ϕadh genome (55) by hybridization. Only one signal, corresponding to the 6-kb *Bgl*II fragment, was detected. The failure of previous attempts to clone this fragment in either *E. coli* or *L. gasseri* ADH (55) most probably was due to its lytic potential, particularly since the vector (pGK12) used in these experiments was not suited to prevent the expression of functions which are detrimental to the cells.

Nucleotide sequence analysis. Nucleotide sequencing showed that both recombinant plasmids carried the identical 1,093-bp *Eco*RI insert of ϕadh DNA (Fig. 1A). This DNA fragment contained an open reading frame (ORF1) of 342 bp, beginning with ATG at position 288 (Fig. 1B), with the potential to encode a protein of 114 amino acids. A second coding region (ORF2) in the same reading frame, starting at an ATG codon separated only by two TAA stop codons from the 3′ end of ORF1, was found to extend beyond the right end of the cloned *Eco*RI fragment (Fig. 1A). Phage ϕadh DNA together with oligonucleotides hybridizing to the DNA sequence established from the 1,093-bp *Eco*RI fragment were then used to complete the ORF2 sequence (951 bp), which can be translated to a protein of 317 residues (Fig. 1B). To eliminate the possibility of base pair changes which may have occurred during the initial PCR amplification of the cloned 1,093-bp *Eco*RI fragments from the lambda gt11 derivatives, the corresponding nucleotide sequence was verified with phage ϕadh DNA as the template. No differences in the DNA sequence were detected.

The sequences 5′ to the putative start codons of ORF1 and ORF2 did not reveal remarkable agreement with promoter consensus sequences reported for gram-positive (15) or gram-negative bacteria (32). Following the stop codon of ORF2, several inverted repeats could be identified. One of them, capable of forming the most stable stem-loop structure (ΔG = −26.8 kcal [ca. −112.1 kJ]/mol), is located between positions 1691 and 1726 in Fig. 1B. It contains two perfectly complementary regions of 11 nucleotides and ends in a run of three T residues (Fig. 2). Whether this stem-loop structure acts as a rho-independent transcriptional terminator (61) remains to be shown. It thus appears that both coding regions belong to one operon which starts beyond the left end of the sequenced DNA fragment (Fig. 1).

Both ORFs are preceded by potential ribosome-binding sites (5′-GAGGAA-3′ for ORF1 and 5′-AAAGAGG-3′ for ORF2) (Fig. 1B) which are similar to the ribosome-binding sequences of the *Lactobacillus* genes tabulated by Alpert and Chassy (1) and of several genes of *Lactobacillus plantarum* (37). They show complementarity with the 3′ ends of the 16S rRNAs of *L. plantarum* (37), *Lactococcus lactis* (11), *B. subtilis* (74), and *E. coli* (68). The spacings between the last G residues

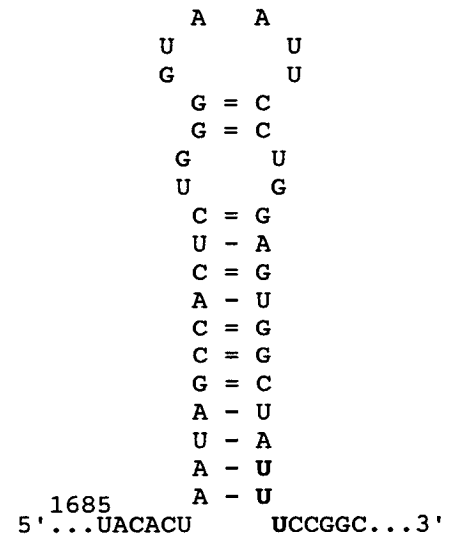
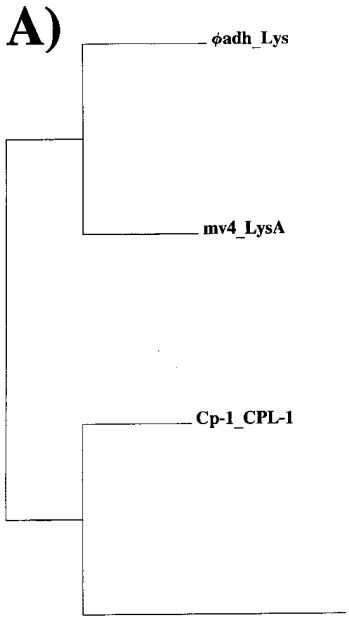


FIG. 2. Potential secondary structure of the mRNA transcribed from the 3′ flanking region of *lys*. Nucleotide numbering is as in Fig. 1B.

of the assigned ribosome-binding sequences and the respective start codons (10 bp for ORF1 and 8 bp for ORF2) fall within the range observed for other *Lactobacillus* sequences (1, 37).

Structural features of the ϕadh lysin. A BLASTX (3) comparison of protein sequences obtained by translation of both strands of the ϕadh DNA sequence in all three reading frames with sequences in protein databases revealed considerable homology of ORF2 to mureinolytic enzymes (lysins) encoded by the *Lactobacillus bulgaricus* phage mv1 (8) (38% identity within 167 residues) and by the *S. pneumoniae* phages Cp-1 (24) (39% identity within 66 residues), Cp-7 (26) (38% identity within 59 residues), and Cp-9 (26) (37% identity within 64 residues). ORF2 was therefore designated *lys* to indicate that it might represent the gene for ϕadh lysin. By using BLASTN (3), none of the nucleic acid sequences encoding these lysins were found to share significant similarity with ϕadh *lys*. The ϕadh *lys* gene, however, has considerable homology (71% within 88 nucleotides) with a region of the yet unpublished sequence of the putative muramidase gene (*lysA*) of *L. bulgaricus* phage mv4 (18). This homology was not detected with *lysA* of the closely related phage mv1, although it is almost identical with *lysA* of mv4 over a length of 867 nucleotides. Because of frameshifts at two positions, representing deletions in *lysA* of mv1 relative to mv4, the region of homology between the ϕadh and mv4 sequences covers the stop codon of the aligned *lysA* gene of mv1. The amino acid sequences derived from the region of highest DNA homology between ϕadh *lys* and mv4 *lysA* are almost identical (94% identity within 17 residues) (Fig. 3B, motif D).

To assess degrees of overall homology, the sequence of the putative *lys* product of ϕadh was compared with the lysin sequences encoded by phage mv4 (LysA) and Cp-1 (CPL-1) and by the *Lactococcus lactis* phage ϕLC3 (LysB). The recently established sequence of the *lysB* product of ϕLC3 was also found to share homology with the mv1, Cp-1, Cp-7, and Cp-9 lysins (5). The dendrogram shown in Fig. 3A was derived from a series of pairwise similarity analyses (20). Among the four lysins compared, those of phage ϕadh and mv4 displayed the highest degree of identity (39.1%) and a similarity of 59.1% when conservative substitutions were taken into account. We also performed a multiple alignment of the lysin sequences



B)

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ϕadh_Lys    MTQTIENRAYGV--------DVSSFNNAN------------VTEYTNAGA   30
mv4_LysA    MTKT-----YGV--------DVAVYQPID------------LAAYHKAGA   25
Cp-1_CPL-1  M---VKKNDL-----FVDVSSHNGYDITG-----------ILEQMGTTNT   31
ϕLC3_LysB   MKRLIKKSAIGMFAFFVVAASGPVFAAVGDQGVDWSKYNGTYGNFGYAHD   50
            *                .   ..  .              ..   . .

ϕadh_Lys    NFVLVKVSE--GLDYRNPKA-KAQVDSTKQNNVVPMGYHYAHFGADSNRA   77
mv4_LysA    SFAIVKLTE--GVDYVNPKG-AKQVDSSRANHLYTHAYHFARFGSSVSRA   72
Cp-1_CPL-1  IIKISE-----STTYLNRC-----LSAQVEQSNPIGFYHFARFGGDVAEA   71
ϕLC3_LysB   KFAFSQIGGTYGGTFVDQATYETQVASAIAQGKRAHTYIWYQVGGSQEVA  100
             . . .    . .. ..      ...  ..      * . . *.. . *
                                                 motif A
ϕadh_Lys    VQEGNYAISSAKLAGVVVGSFLACDYEQGSGNETRGDREANTTAILAFLD  127
mv4_LysA    KKEAAYFLKEAKKQDISKKRMLWLDWEAGSGNVVTGSKSSNTAAILDFMD  122
Cp-1_CPL-1  EREAQFFLDNVPMQ---VKYLV-LDYEDD----PSGDAQANTNACLRFMQ  113
ϕLC3_LysB   KAALDRYLPKIQTP---KNSIVALDYEGG----ASGNKQANTDAILYGMR  143
              . .  . .   .      ..  *.* .      *.  .**.* *  .
              motif B                        motif C
ϕadh_Lys    TIVSAGYKPLLYSGAYLMKNKINTSRILAKYPDCLWVAAYPLGNGVSANV  177
mv4_LysA    AIKAAGWRPGLYSGASLMRTAIDTKQVVKKYGTCLWVASYPTMAAVST--  170
Cp-1_CPL-1  MIADAGYKPIYYSYKPFTHDNVDYQQILAQFPNSLWIAGYGLNDG--TA-  160
ϕLC3_LysB   RVKAAGYTPMYYSYKPYTLANVNYKQIIKEFPKSLWIAAYPNYEV--TPV  191
             . .**. *  **      . ..  ... .. ..**.*.*   .   .
                motif D
ϕadh_Lys    PNFEYFPSMDGVAIWQFTDNWKGMNVDSNIAVKSLSFNFNVAKPASQAVK  227
mv4_LysA    ADFGYFPSMDGVAIWQFTSNWHGLDVDGNVALVDLN---SENKPKAEVKP  217
Cp-1_CPL-1  -NFEYFPSMDGIRWWQYSSN----PFDKNIVLL---------DDEEDDKP  196
ϕLC3_LysB   PNYSFFPSMDGISVFQFTSTYVAGGLDGNVDLTGITGNGYGKQKGQEVKP  241
             ....******.  .*....      * *. .        .   .

ϕadh_Lys    TST-----QPKTWTDVQGMN-----------------WYEEYGTFITGGA  255
mv4_LysA    KTK-----QKATATDFHGVV-----------------KVKSLGA--SKAS  243
Cp-1_CPL-1  KTAGTWKQDSK------------GWWFRRNNGSFPYNKWEKI-----GGV  229
ϕLC3_LysB   NTATPAIENGKEANEVKGNDVEVGMTVKVNFGAKNYATGETIPQWVKGQP  291
            ..      .                              ..

ϕadh_Lys    IN--------------LRWGATTQSSIIAQL---PAGVEVKY--------  280
mv4_LysA    WK--------------VRLLSKDGHYTESYV---PQGSR-----------  265
Cp-1_CPL-1  WYYFDSKGYCLTSE----WLKDNE---------------KWYYLKDNGAM  260
ϕLC3_LysB   HKIIQKNGDTVLLDGIMSWLSVHDVETIDASTSQPTTPAKSYIVKQGDTL  341

ϕadh_Lys    ---------------------DAWSRDQAGRVWLRQPR----ANGYGYLV  305
mv4_LysA    ----------------------WKTSEVTTIKGKKC----------YLI  282
Cp-1_CPL-1  ATGWVLVGSEWYYMDDSGAMVTGWVKYKNNWYYMTNERGNMVSNEF-IKS  309
ϕLC3_LysB   SGIASNLGTNWQELARQNSLSNPNMIYSGQVISLTGGQSGATARTYTVQS  391

ϕadh_Lys    GR---------------------VGNEAW-----GTFK  317
mv4_LysA    GKDL----W--------------IPEEFV-----TITK  297
Cp-1_CPL-1  GK-----GWYFMNTNGELADNPSFTKEP---DGLITVA  339
ϕLC3_LysB   GDNLSSIARRLGTTVQSLVSMNGISNPNLIYAGQTLNY  429
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FIG. 3. Comparison of ϕadh lysin with murein hydrolases encoded by other phage of gram-positive bacteria. (A) Dendrogram of the deduced amino acid sequences of the lysins of *L. gasseri* phage ϕadh (ϕadh_Lys), *L. bulgaricus* phage mv4 (18) (mv4_LysA), *S. pneumoniae* phage Cp-1 (23) (Cp-1_CPL-1), and *Lactococcus lactis* phage ϕLC3 (5) (ϕLC3_LysB). Relationships were calculated by the TREE program (Husar). The lengths of the horizontal branches directly correlate with the frequencies of amino acid exchanges in the respective proteins. (B) Multiple alignment of the lysins depicted in panel A. The alignment was performed with the CLUSTAL program (36) by using a gap penalty of 5 and a window size of 10. Perfectly conserved residues are marked with asterisks below the sequences; dots indicate positions with conservative substitutions. Four regions, displaying 45.5% (motif A), 50% (motifs B and C), and 46.7% (motif D) identity of the four sequences are indicated. Sequences are designated as in panel A. Residue numbers are shown on the right.

(Fig. 3B). Four regions (motifs A through D) of remarkably high identity (>45%) can be identified. Although none of these motifs displayed similarity to any of the sequence patterns and sites deposited in the PROSITE dictionary (4), their conservation strongly suggests that they are of structural or functional significance.

Unlike the muramidases of phage Cp-1, Cp-7, and Cp-9 (26) and the pneumococcal major autolysin (25), the ϕadh lysin does not contain C-terminal homologous repetitions. Such sequence repeats of 20 or 48 amino acids have been implicated in substrate recognition by muramidases (26). The lysin of ϕadh has an internal region of 17 amino acids (Fig. 1B) which, according to the algorithm of Klein et al. (43), is predicted to form a transmembrane segment. The positive charge of a Lys within this region, apparently militating against this prediction, may be compensated by a preceding Glu which is located eight amino acids away. Potential transmembrane domains have thus far not been identified in the murein hydrolases encoded by other phages.

Structural features of the ϕadh holin. Computer-assisted sequence comparisons did not reveal any significant homologies of ORF1 or its predicted protein product with entries in the available databases. Functional complementation of the *S*am mutation of lambda gt11 by ORF1 together with its location upstream of *lys* strongly suggested that it might encode a holin. The ϕadh ORF1 product does in fact share some striking structural features with the holins of other phage (78). It has a pair of potential transmembrane domains (i.e., regions of at least 20 residues with no net charge) (Fig. 1B), separated by a sequence which is likely to form a β turn (12). Using the algorithms of Klein et al. (43) and von Heijne (75), respectively, we identified the first of these domains as both a potential transmembrane helix and a possible signal sequence. The second predicted transmembrane domain contains one negative and one positive charge which, because of the spacing of seven residues, may be located next to each other in the helix. Moreover, the ORF1 product is highly hydrophobic (average hydropathy = −0.57) (44), its calculated isoelectric point is basic (9.52), and it possesses a charged C terminus. On the basis of these structural similarities with previously described holins, ORF1 was termed *hol.*

Codon usage. The overall G+C content of the sequenced DNA region (1,735 bp) of phage ϕadh was calculated to be 38.3%, which is slightly higher than the value (34%) observed for chromosomal DNA of its host *L. gasseri* (41). Presumably because of this rather low value, the codons of ϕadh *hol* and *lys*, in contrast to some genes from *Lactobacillus casei* (46, 52) and *Lactobacillus delbrückii* subsp. *bulgaricus* (65), do not show a preference for G and C in the third position. The G+C contents in position 3 (20.2% for *hol* and 25.9% for *lys*) are even lower than those of the entire genes (32.8% for *hol* and 39.8% for *lys*). Eccentric usage of the Arg codons AGA and AGG was observed. These triplets, which were previously reported to be absent from a series of *Lactobacillus* genes (46), constitute 100 and 50% of the Arg codons of *hol* and *lys*, respectively. This seems to rule out the notion that AGA and AGG may function as stop codons in lactobacilli (46), as it is the case in the mitochondria of vertebrates (23). Moreover, the codon ATA (Ile), known to be extremely rare in genes of

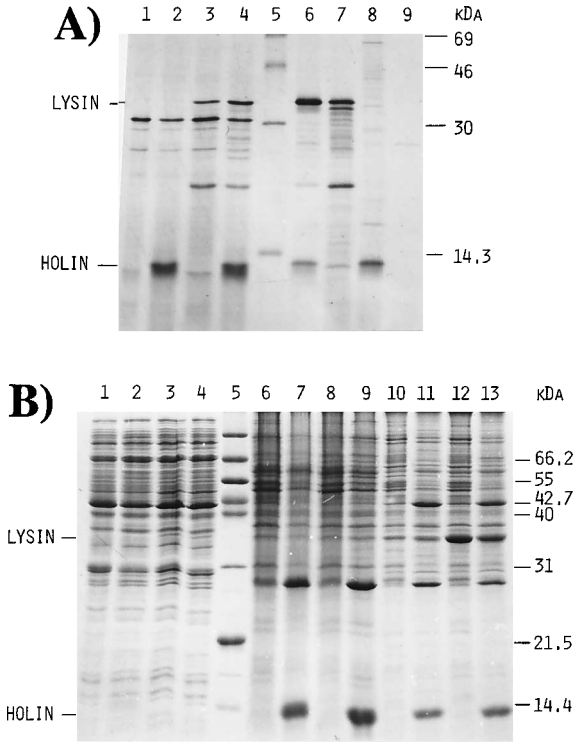

FIG. 4. Identification and localization of φadh *hol* and *lys* products. (A) Plasmid-encoded, ^{35}S-labelled proteins were synthesized in vitro with a DNA-directed translation system in the presence of plasmids pUC19 (lane 1), pUC19hol (lane 2), pUC19lys (lane 3), and pUC19holys (lane 4) or in vivo by infecting *E. coli* MC4100I^{Q} harboring plasmids pSKholys (lane 6), pSKlys (lane 7), pSKhol (lane 8), and pBluescriptSK+ (lane 9) with lambda CE6 (see Materials and Methods). Aliquots of the in vitro reactions and of the lauryl sarcosinate-soluble membrane fractions prepared from the in vivo samples were analyzed on a sodium dodecyl sulfate–16.5% polyacrylamide gel. The positions and molecular masses of protein standards (lane 5) are indicated at the right. (B) The cytoplasmic fractions (lanes 1 to 4), the cytoplasmic membrane fractions collected from sucrose step gradients (lanes 6 to 9), and the lauryl sarcosinate-soluble membrane fractions (lanes 10 to 13) were prepared from transformants of *E. coli* MC4100I^{Q} grown in the presence of IPTG (see Materials and Methods). Plasmids used were pUC19 (lanes 1, 6, and 10), pUC19hol (lanes 2, 7, and 11), pUC19lys (lanes 3, 8, and 12), and pUC19holys (lanes 4, 9, and 13). Proteins were separated on a sodium dodecyl sulfate–16.5% polyacrylamide gel and visualized by staining with Coomassie blue. The positions and sizes of protein standards (lane 5) are indicated on the right.

various *Lactobacillus* species (46), appears once in *hol*, whereas it is absent from *lys*. The codon CTG (Leu), frequently used in *E. coli* (66) but absent from a number of *L. casei* genes (73), is not present in either φadh lysis gene.

Identification and subcellular location of the φadh *hol* and *lys* products. Plasmids containing the isolated gene *hol* or *lys* or the continuous *hol-lys* sequence together with their respective ribosome-binding sites under transcriptional control of either the *lac* promoter (derivatives of pUC19) or the T7 promoter (derivatives of pBluescriptKS+) were used for electrophoretic identification of the φadh lysis proteins. The pUC19 derivatives were used as templates in a coupled in vitro transcription-translation system (Fig. 4A, lanes 2 to 4). As judged from the electrophoretic mobilities of the synthesized proteins, the apparent molecular masses of 13.5 kDa for the holin and 34.5 kDa for the lysin agreed well with the values of 12.886 and 34.706 kDa calculated from the predicted amino acid sequences. The pBluescriptSK+ derivatives were used to express the cloned genes in vivo by infection of the respective transformants with phage lambda CE6 delivering the T7 RNA polymerase (lanes 6 to 8). Since the in vitro- and in vivo-synthesized products of *hol* and *lys* showed the same electrophoretic mobilities, posttranslational modifications, such as cleavage of the potential signal sequence of the φadh holin (Fig. 1B), are rather unlikely. Expression of the *lys* gene resulted also in the production of a protein with an apparent molecular mass of approximately 20 kDa. As the corresponding band can be detected among both the in vitro- and in vivo-labelled proteins (lanes 3, 4, 6, and 7), it may originate from translation initiation within *lys* rather than from degradation of the lysin. A candidate for the initiation of an in-frame product of 173 amino acids with a predicted molecular mass of 19.3 kDa is the Met codon at position 1068, which is preceded by a possible ribosome-binding site (5′-AGGAG-3′) (Fig. 1B).

To determine the subcellular location of the φadh lysis proteins in *E. coli*, transformants expressing the *hol* and *lys* genes from the respective pUC19 derivatives were subjected to cell fractionation. As shown in Fig. 4B, the φadh holin was detected in the cytoplasmic membrane (lanes 7 and 9) and could be extracted from the total membrane fraction with lauryl sarcosinate (lanes 11 and 13), whereas it was absent from the cytoplasmic fraction (lanes 2 and 4). The visualization of the φadh holin by Coomassie blue staining is rather unusual since holins are in general poorly expressed and thus far have been detected only with the aid of specialized expression systems (2, 7, 70). One or two additional proteins with apparent molecular masses corresponding to the two- or threefold size of the holin appeared concomitantly with the expression of *hol* (lanes 7, 9, 11, and 13). They may represent incompletely denatured dimers or trimers of the φadh holin, which would parallel the detection of oligomeric forms of lambda S protein in the membrane fractions of *Saccharomyces cerevisiae* (28) and *E. coli* (80).

A protein with the predicted molecular mass of the φadh lysin appeared as a faint band among the cytoplasmic proteins (Fig. 4B, lanes 3 and 4). This protein could not be identified in the cytoplasmic membrane (lanes 8 and 9), nor was it detectable in the outer membrane fraction (not shown). However, a band with the expected mobility was clearly enhanced in the pattern of lauryl sarcosinate-soluble total membrane proteins (lanes 12 and 13). The corresponding protein most probably is φadh lysin, since a band with the same mobility was also detected in the lauryl sarcosinate-soluble membrane fraction obtained after selective expression of *lys* or *hol-lys* from the T7 promoter (Fig. 4A, lanes 6 and 7).

φadh holin and lysin are functional in *E. coli*. Using the respective pUC19 derivatives, we tested the effects of expression of the individual *hol* and *lys* genes and of the φadh lysis cassette (*hol-lys*) on the growth of *E. coli*. As shown in Fig. 5A, growth inhibition was observed about 50 min after induction of gene *hol*. Concomitantly, the increase of viable cells arrested. Contrary to the bactericidal effect of the expression of the cloned genes *S* of lambda (29) and *14* of φ29 (70), growth retardation after induction of φadh *hol* does not suggest a serious membrane lesion in *E. coli*. Expression of the φadh *lys* gene had no apparent effects on growth rate or cell viability, whereas addition of chloroform to the induced culture resulted in immediate lysis (Fig. 5B). This finding is consistent with other studies which showed that overexpression of lambda gene *R* (29) or P22 gene *19* (58), in the absence of the respective holin functions, does not affect growth of *E. coli*. The dramatic effect of chloroform, previously shown to induce premature lysis of lambda-infected cells by permitting access of the R protein to the murein (27), demonstrates that the φadh lysin serves a mureinolytic function and that it is operational in *E. coli*. However, when the φadh *hol* gene was expressed to-

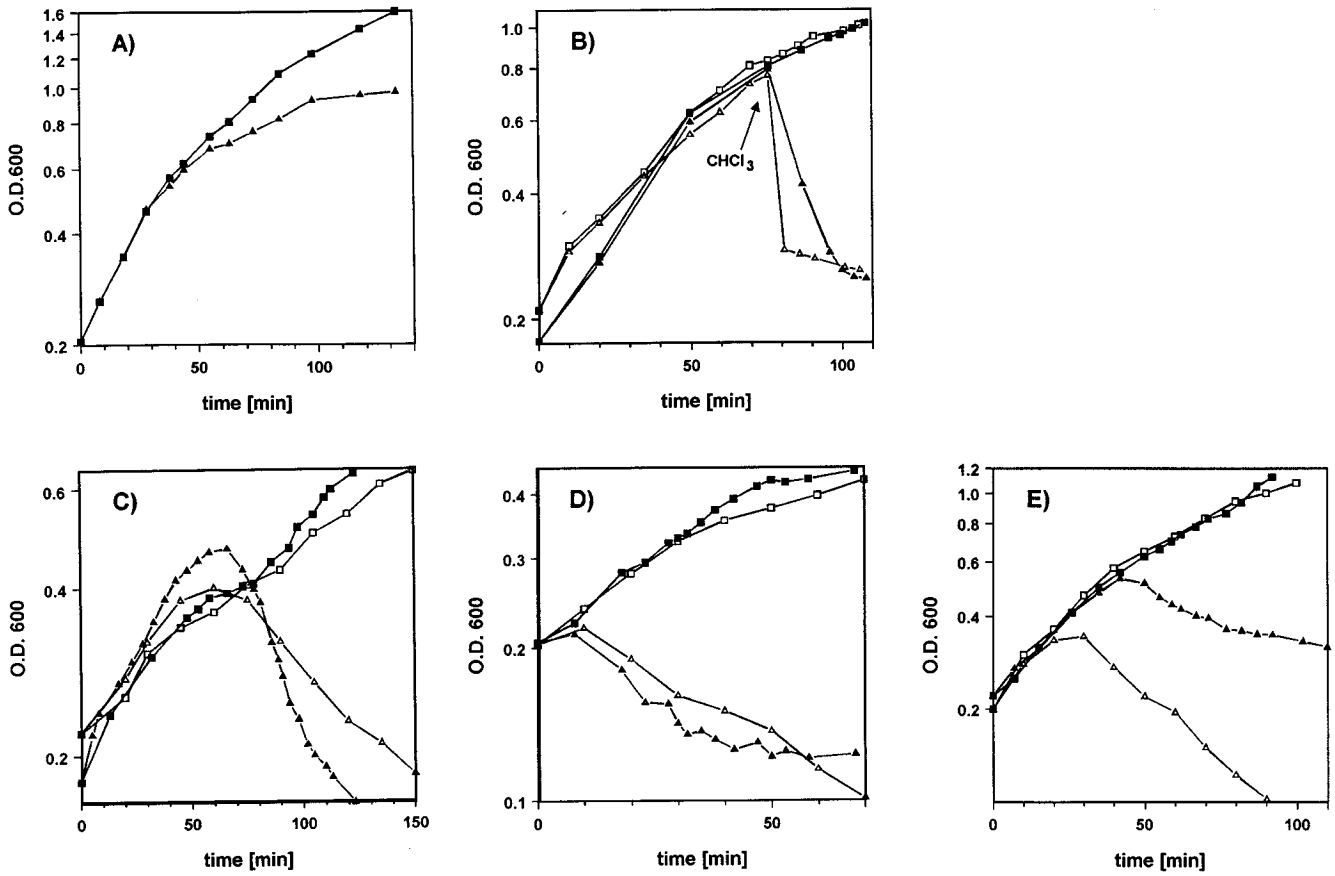

FIG. 5. Coexpression of ϕadh lysis genes with complementary functions from other phage. Transformants of *E. coli* were grown at 37°C (or 28°C for lambda lysogens) in LB broth supplemented with appropriate antibiotics. At time zero, transcription of cloned lysis genes was induced in aliquots of the cultures (▲, △) by addition of IPTG (5 mM, final concentration) and, in the case of lambda lysogens, by simultaneous temperature shift to 42°C for 1 h followed by further incubation at 37°C. ■, □, uninduced controls. Lysis functions of various phage were coexpressed by use of the following combinations of strains and plasmids: (A) MC4100I^{Q} with pUC19hol (ϕadh *hol*) (▲); (B) MC4100I^{Q} with pUC19lys (ϕadh *lys*) (▲) or pUC19holys (ϕadh *hol-lys*) (△) (at the time indicated by the arrow, $CHCl_3$ [2%, final concentration] was added to the induced cultures); (C) MC4100I^{Q}::lambda 111 with pUC19hol (lambda *R* plus ϕadh *hol*) (▲) or pUC19holys (lambda *R* plus ϕadh *hol-lys*) (△); (D) MC4100I^{Q}(pSB29-2) with pUC19hol (ϕ29 *15* plus ϕadh *hol*) (▲) or pUC19holys (ϕ29 *15* plus ϕadh *hol-lys*) (△); (E) MC4100I^{Q}(pSB29-11) with pUC19lys (ϕ29 *14* plus ϕadh *lys*) (▲) or pUC19holys (ϕ29 *14* plus ϕadh *hol-lys*) (△). O.D.600, optical density at 600 nm.

gether with the authentic *lys* gene from a single plasmid (pUC19holys), the effects on cell growth and viable cell counts were very similar to those obtained by the sole expression of the *hol* gene (not shown). In contrast to other systems (27, 70), no signs of lysis could be detected at either 37 or 42°C. We therefore cloned both the *hol* and *lys* genes separately under the *lac* promoter of the vector pK194. The pK194 derivatives were introduced into *E. coli* clones carrying the complementary lysis functions of ϕadh on the compatible plasmids pUC19lys and pUC19hol, respectively. Efforts to induce lysis in the resulting double transformants also failed, irrespective of the combination of the pK194 and pUC19 derivatives used.

Holins and lysins from different phage are interchangeable. Using a lambda lysogen and derivatives of plasmids pK184 and pUC19, we expressed holin and lysin genes of unrelated phage of gram-negative and gram-positive bacteria in *E. coli* concomitantly with the complementary lysis genes of phage ϕadh. Induction of the lambda *R* gene, either from a lambda lysogen (MC4100I^{Q}::lambda 111) (Fig. 5C) or from a plasmid (pKS^{-}R^{+}) (not shown), was followed by lysis after about 50 min. Functional similarity of the ϕadh holin and the lambda S protein was also demonstrated by the observation that the plating efficiency of lambda gt11 (*S*100) on MC4100I^{Q} (pUC19hol) was 5,000-fold higher than on MC4100I^{Q}(pUC19 dtac). Taking into account a 15-fold increased plating efficiency observed with lambda gt11 on the *supE supF* strain Y1088 relative to MC4100IQ(pUC19hol), complementation of lambda *S*100 by ϕadh *hol*, however, was rather inefficient. The lysin of *B. subtilis* phage ϕ29 (protein 15), expressed from plasmid pSB29-2, appears to be released very efficiently to the periplasm by the action of ϕadh holin, since rapid lysis occurred within 10 min after induction (Fig. 5D). Even the low-level constitutive expression of phage T7 gene *3.5* (encoding an amidase) from plasmid pLysS was sufficient to cause slow cell lysis about 100 min after induction of the ϕadh *hol* gene (not shown). These effects indicate that the lesions induced by ϕadh holin in the cytoplasmic membrane of *E. coli* are nonspecific. Figure 5E illustrates the effect of simultaneous expression of phage ϕ29 protein 14 (holin) from plasmid pSB29-11 and the ϕadh lysin from pUC19lys in *E. coli*. The culture lysed 40 min after induction. This experiment showed that the ϕadh lysin can be released to the murein through the action of ϕ29 protein 14.

Since the expression studies (Fig. 4) clearly demonstrated that both the holin and the lysin are produced with plasmid pUC19holys, their failure to bring about lysis of *E. coli* raised the question of whether the pUC19holys-encoded lysis proteins are functional. Premature lysis occurred when chloroform

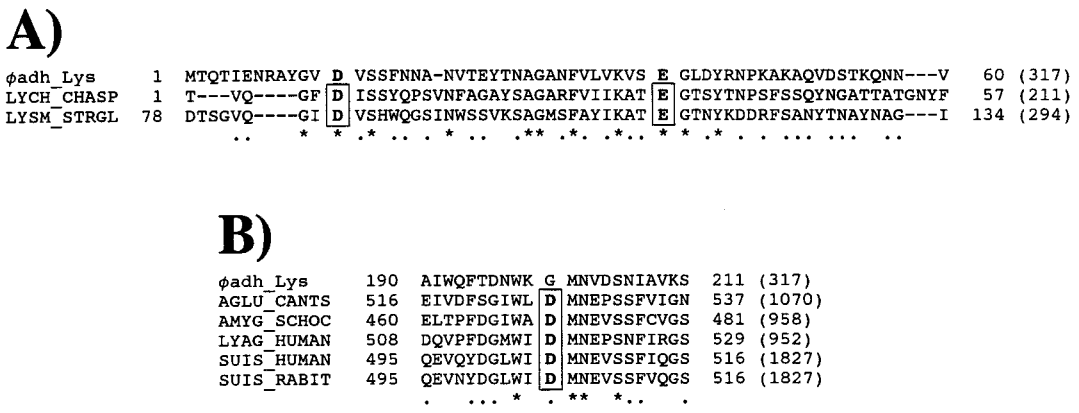

FIG. 6. Comparison of ϕadh lysin (ϕadh_Lys) with catalytic domains of lysozyme Ch of *Chalaropsis* sp. (19) (LYCH_CHASP) and *N*-acetylmuramidase M1 of *Streptomyces globisporus* (47) (LYSM_STRGL) (A) and of α-glucosidase from *Candida tsukunbaensis* (42) (AGLU_CANTS), glucoamylase 1 from *Schwanniomyces occidentalis* (50) (AMYG_SCHOC), lysosomal α-glucosidase from humans (34) (LYAG_HUMAN), and intestinal sucrase-isomaltases from humans (10) (SUIS_HUMAN) and rabbits (38) (SUIS_RABIT) (B). Numbers of the first and last residues of the aligned regions and the total lengths of the sequences (in parentheses) are given. Perfectly conserved residues are marked with asterisks; dots indicate positions with conservative substitutions. Essential residues in the active sites of individual sequences are boxed.

was added after induction of *hol-lys* (Fig. 5B), and concomitant expression of *hol-lys* with the lambda transglycosylase, with the ϕ29 lysozyme, and with the ϕ29 holin in all cases resulted in cell lysis (Fig. 5C to E). This finding clearly shows that both of the ϕadh lysis genes function individually when expressed from plasmid pUC19holys.

DISCUSSION

Structural and functional properties of phage-encoded lysis proteins are conserved in ϕadh. It has been previously noted that all of the characterized holin proteins apparently permit a nonspecific release of murein hydrolases to the periplasm (58, 70) and appear to function in heterologous systems (28, 70). Since the ϕadh *hol* gene can complement the lambda *S* gene, the experiments described here lend further support to this notion. The nonspecific release of unrelated murein hydrolases by ϕadh holin (Fig. 5C and D), together with its subcellular localization in the membrane fraction (Fig. 4B) and its primary structure (Fig. 1B), which displays traits typical for other holin proteins (basic isoelectric points, high hydrophobicity, predicted pairs of transmembrane domains separated by β turns, absence of a net charge in either transmembrane domains, and highly charged C-terminal ends) (78), clearly classify the ϕadh *hol* gene as a holin function. Moreover, ϕadh *hol* maps immediately upstream of the *lys* gene, which agrees with the arrangement of the analogous holin and murein hydrolase genes in phage lambda (13), P22 (58), 21 (9), and ϕ29 (30), respectively. On the basis of structural similarities of derived proteins, putative holin genes can also be identified upstream of the lysin genes of other phage of gram-positive bacteria like *S. pneumoniae* phage EJ-1 (ORF2) (16) and *Lactococcus lactis* phage ϕUS3 (51).

Regardless of the structural resemblance, the ϕadh holin displays no sequence relatedness to any of the previously characterized or inferred holins (5, 16, 78). Since the holins thus far tested show no specificity for the authentic lysins (58, 70), it seems reasonable to attribute their similarity in function to a common structural architecture rather than to their primary sequences.

Why do the combined *hol* and *lys* functions of ϕadh not effect lysis of *E. coli*? Concomitant induction of both the ϕadh *hol* and *lys* genes from plasmid pUC19holys was followed by a cessation of growth rather than by cell lysis. This observation contrasts recent studies showing that the ϕ29 holin (protein 14) (70) as well as the ϕLC3 LysA holin (5) mediate transit of ϕ29 and ϕLC3 lysins, respectively, to the periplasm of *E. coli*. Thus, the observations concerning the ϕadh lysis system appear somewhat contradictory to our current concept of holins, which would predict that a genuine holin permits the transit of any lysin to the periplasm. In fact, the ϕadh lysin was apparently released by the ϕ29 holin (protein 14) (Fig. 5E). Why does the ϕadh holin-lysin system not work in *E. coli* even though both proteins are well expressed from the *lac* promoter (Fig. 4B) and either one of the isolated or combined lysis functions behaves just as predicted when expressed along with a heterologous holin or lysin (Fig. 5)? Possible reasons might be related to weak activity of the lysin or to inaccurate interaction of both lysis proteins in the *E. coli* system. Weak activity cannot be imputed to the lysin, since chloroform liberated sufficient mureinolytic activity from MC4100I^{Q}(pUC19holys) to provoke dramatic cell lysis (Fig. 5B). On the other hand, the observed release of both the lambda and ϕ29 lysins by the ϕadh holin would not suggest a specific interaction with these proteins. It thus may be speculated that some additional signal or event, absent in *E. coli*, is necessary for the ϕadh holin-mediated transit of the cognate lysin across the membrane and for the regulation of the lysis event. In the presence of heterologous lysis functions, the regulatory interactions may not occur.

In contrast to previously characterized phage-encoded murein hydrolases, which have been exclusively detected in the cytoplasm (78), the bulk of the ϕadh lysin was pelleted together with the total membrane fraction of *E. coli*. Although it could be extracted from this fraction with lauryl sarcosinate, it was not detectable in either of the inner and outer membrane preparations obtained by separation of the total membranes in a sucrose step gradient. Two possibilities accounting for these observations can be imagined. Either the ϕadh lysin is merely insoluble because of heavy overexpression in *E. coli*, or it is actually associated with the membrane fraction through weak interactions, which cannot withstand the physical separation of the inner and outer membranes. It remains to be elucidated whether the unusual localization of the ϕadh lysin in *E. coli* is of any importance for its release to the murein in the *Lactobacillus* system.

Sequence similarity suggests that ϕadh lysin is a muramidase. Looking for a signature that would point to the catalytic site of ϕadh lysin, we compared its sequence with the N terminus of lysozyme Ch of the fungus *Chalaropsis* sp. (19), which was previously shown to be similar to the N termini of muramidase CPL-1 of *S. pneumoniae* phage Cp-1 (24), *N*-acetylmuramidase M1 of *Streptomyces globisporus* (47), and LysA of *L. bulgaricus* phage mv1 (8). Lysozyme Ch, CPL-1, and M1 all contain an Asp and a Glu residue 26 or 27 amino acids apart, which, in the case of the *Chaloropsis* enzyme, were shown to be involved in catalytic activity (22). This is consistent with the observation that the isolated N termini of the Cp-1, Cp-7, and Cp-9 lysozymes exhibit mureinolytic activity (64). The sequence of phage ϕadh lysin can also be aligned with lysozyme Ch and *N*-acetylmuramidase M1 in such a way that Asp-13 and Glu-39 appear as conserved residues and that the intermediate and flanking residues display a significant degree of similarity (Fig. 6A). As the muramidase activities of lysozyme Ch (31) and *N*-acetylmuramidase M1 (77) have been demonstrated biochemically, this could indicate that ϕadh lysin acts through a similar catalytic mechanism and that its active center is located at the N-terminal end. Another region in the central part of ϕadh lysin (positions 190 to 211), identified by the BLIMPS (Husar) program, appears to share homology with a sequence pattern centered around the active site of a family of glycosyl hydrolases from yeasts (42, 50), humans (10, 34), and rabbits (38). Figure 6B shows a similarity of 55% and four perfectly conserved residues in the aligned region. The Asp residue, reported to be located in the catalytic site of the glycosyl hydrolases, however, is exchanged for Gly in the sequence of ϕadh lysin. Since replacement of Asp by Gly in the catalytic center of human lysosomal α-glucosidase resulted in loss of activity (34), the significance of the observed similarity to ϕadh lysin remains questionable.

ACKNOWLEDGMENTS

We are grateful to Eric Altermann, Heike Litzenburger, and Bernd Winkelmann for their friendly and reliable assistance, to Tonja Schmidt for the gift of ϕadh DNA, and to Karsten Tedin for comments on the manuscript.

B.B. was supported in part by fellowship 940867 from the Austrian Ministry of Science and Research. This work was funded in part by grant P10006-MOB from the Austrian Science Foundation to U.B.

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