In the cycle of vegetative reproduction of the temperate bacteriophage P1, a number of late functions become activated by the product of gene 10 (16, 18). Two phage-specific late promoter sequences, Pd and Pdar, from which the tail-fiber promoter by the product of gene lydB of bacteriophage P1, a number of late functions become activated and exhibited no known extended homology. To study the effect of gpl7, LydA, and LydB in vivo, their genes were cloned in a single operon under the control of the inducible T7 promoter, resulting in plasmid pAW1440. A second plasmid, pAW1442, is identical to pAW1440 but has lydB deleted. Induction of the T7 promoter resulted in a rapid lysis of cells harboring pAW1442. In contrast, cells harboring pAW1440 revealed only a small decrease in optical density at 600 nm compared with cells harboring vector alone. The rapid lysis phenotype in the absence of active LydB suggests that this novel protein might be an antagonist of the holin LydA.

Three Functions of Bacteriophage P1 Involved in Cell Lysis

CHRISTOPH SCHMIDT,1 MATHIAS VELLEMAN,2 AND WERNER ARBER1*

Abteilung Mikrobiologie, Biozentrum der Universität Basel, CH-4056 Basel, Switzerland, and Abteilung Schuster, Max-Planck-Institut für Molekuleare Genetik, D-14195 Berlin, Germany.

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Amber and deletion mutants were used to assign functions in cell lysis to three late genes of bacteriophage P1. Two of these genes, lydA and lydB of the dar operon, are 330 and 444 bp in length, respectively, with the stop codon of lydA overlapping the start codon of lydB. The third, gene 17, is 558 bp in length and is located in an otherwise uncharacterized operon. A search with the predicted amino acid sequence of LydA for secondary motifs revealed a holin protein-like structure. Comparison of the deduced amino acid sequence of gene 17 with sequences of proteins in the SwissProt database revealed homologies with the proteins of the T4 lysozyme family. The sequence of lydB is novel and exhibited no known extended homology. To study the effect of gpl7, LydA, and LydB in vivo, their genes were cloned in a single operon under the control of the inducible T7 promoter, resulting in plasmid pAW1440. A second plasmid, pAW1442, is identical to pAW1440 but has lydB deleted. Induction of the T7 promoter resulted in a rapid lysis of cells harboring pAW1442. In contrast, cells harboring pAW1440 revealed only a small decrease in optical density at 600 nm compared with cells harboring vector alone. The rapid lysis phenotype in the absence of active LydB suggests that this novel protein might be an antagonist of the holin LydA.

In this study, we cloned and sequenced the bacteriophage P1vir genes 17, lydA, and lydB as well as their alleles in the P1vir amber mutants am17.20 and am2.31. On the basis of sequence comparisons and studies of their activities in an in vivo expression assay, the functions of these genes were found to be consistent with the functions previously assigned to them: gp17 is a lysozyme and LydA is a holin, of which LydB is an antagonist of a novel type.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The E. coli K-12 strains used in this study were UT580 supD hisZ (13) and JM101 supF lac-pro (32). The thermoinducible phage P1-15:Ts2680 (20), carrying the c1ts225 allele and a kanamycin resistance marker, and the amber mutants P1vir am17.20 and am2.31 were used.

Plasmid pMC4.1 (6) is a pBR325 derivative harboring the P1 BamHI:4.1 restriction fragment, which is the larger of the two BamHI-AclI restriction fragments. Plasmid pUM4.1 (6) is a pUC19 derivative harboring the 0.9-kb KpnI-HincII subfragment of pMC4.1. The low-copy-number plasmid pWSK30 (31), a Bluescript II KS+ (Stratagene) derivative which has ColEl replaced by the pSC101 origin of replication and confers ampicillin resistance, was used as a vector to clone lydA, lydB, and gene 17. Plasmid pGPI-2 (27), which confers kanamycin resistance, is a pACYC177 derivative harboring the heat-inducible T7 RNA polymerase gene. Plasmids constructed in this work are described below.

Growth media. Standard liquid media and agar plates as described by Maniatis et al. (19) were used for the propagation of bacteria and phages. Selective media contained appropriate combinations of the following antibiotics: 100 μg of ampicillin per ml and 25 μg of kanamycin per ml (final concentrations).

DNA techniques. Standard DNA techniques were carried out as described by Maniatis et al. (19). DNA size markers for gel electrophoresis were purchased from Bethesda Research Laboratories.

Cloning of P1 subfragments. The 195-bp KpnI-ApoI restriction fragment from pUM4.1, harboring the Lpr21 late promoter sequence, was cloned into the KpnI-EcoRI restriction sites of pUC19 (32), resulting in pAW1438. Plasmid pAW1440, which harbors two different fragments from phage P1, was constructed as follows. The 0.9-kb EcoRI-HindIII restriction fragment from pUM4.1, harboring gene 17, was cloned into the EcoRI-HindIII restriction sites of pWSK30. Additionally, the blunt-ended 0.9-kb ApaI-Apel restriction fragment from the P1 Smal-L fragment harboring the lydA and lydB genes was cloned into the HincII site of pWSK30. In pAW1440, the genes of both fragments are in an
orientation opposite to that of the P_L promoter and hence under the control of the inducible T7 promoter. The 1.5-kb BamHI restriction fragment from pAW1440 was cloned into the BamHI site of pWSK30, resulting in pAW1442. Plasmids pAW1442 and pAW1440 are isogenic, except for the lydB gene, which has been deleted from pAW1442 downstream of the BamHI restriction site.

RNA extraction and primer extension. A 4-ml culture of UT580 (P1-15::Tn2680) carrying plasmid pAW1438 was grown at 30°C in Luria-Bertani medium to an optical density at 600 nm of 0.2. The prophage was then induced to lytic growth by a shift of the temperature to 42°C. After a further 30 min of incubation, total cellular RNA was isolated by the procedure described by Chomczynski and Sacchi.

Primer extension reactions, with the 32P-end-labeled oligonucleotide PNM (5’-GGGTTTTCCCAGTCACGACGTTGT-3’) being used as primer for avian myeloblastosis virus reverse transcriptase (Promega), were carried out according to the method of Geliebter et al. (9). The oligonucleotide hybridizes within the lacZ sequence in pAW1438 approximately 80 bp downstream of the LPr21 transcription initiation site.

Computer analysis. For sequence comparisons, the software package of the Genetics Computer Group was used.

Nucleotide sequence accession numbers. The nucleotide sequences for P1vir between map positions 21 and 20 and between positions 32 and 31 have been assigned GenBank/EMBL accession numbers X87673 and X87674, respectively.

RESULTS

Mapping, cloning, and sequence analysis of lydA and lydB. The lydA and lydB genes had been located previously in the 5’-end of the dar operon around map position 32 (Fig. 1) (14, 34). The 854-bp nucleotide sequence of the AgeI-XbaI restriction fragment containing the late promoter LPdar and the 5’-end of the dar operon was determined (Fig. 2). It includes the previously determined nucleotide sequences of the LPdar promoter and the GTG start codon of lydA (positions 1 to 60) (11) and of the almost identical p15B lydA and lydB (only 5’ end) genes (positions 58 to 567) (24). Two open reading frames, lydA and lydB, from positions 58 to 387 and 384 to 827, respectively, are present, with the start codon of lydB overlapping the stop codon of lydA. For each gene, a putative ribosome binding site.
with the distal end 6 bp upstream of the start codon can be found (not specifically marked in Fig. 2).

To address the function of these genes, the nucleotide sequences of their alleles in the P1 vir sequences of their alleles in the P1 found (not specifically marked in Fig. 2). For example, the distal end 6 bp upstream of the start codon can be found.

In Fig. 3, two base changes in two consecutive codons at coordinates 394 and 336 were found. The fourth codon of lydB, which codes for tryptophan (TGG), was mutated to an amber stop codon (TAG), and the glycine codon (GGT) at position 5 was mutated to a tryptophan (TGG) codon. The two closely linked G-to-A transitions are likely to have resulted from the repair process following the hydroxylamine mutagenesis to produce amber mutations (29, 30). Gene rescue experiments with the gene lydB of phages (35) tested to a serine codon (AGT). The two closely linked G-to-A transitions are likely to have resulted from the repair process following the hydroxylamine mutagenesis to produce amber mutations (29, 30). Gene rescue experiments with the gene lydB of phages (35) tested to a serine codon (AGT). The two closely linked G-to-A transitions are likely to have resulted from the repair process following the hydroxylamine mutagenesis to produce amber mutations (29, 30). Gene rescue experiments with the gene lydB of phages (35) tested to a serine codon (AGT). The two closely linked G-to-A transitions are likely to have resulted from the repair process following the hydroxylamine mutagenesis to produce amber mutations (29, 30). Gene rescue experiments with the gene lydB of phages (35) tested to a serine codon (AGT).

A search for secondary structure motifs in the deduced amino acid sequence of lydA and lydB revealed two putative membrane-spanning domains separated by a β-turn in the amino-terminal part of LydA (Fig. 2), but no such domains were found in LydB. As shown for the phage λ S and S' gene products SI05 and SI07, these membrane-spanning domains are crucial for the hole-forming capability of these proteins (21). They are also found in lysis functions of many other types of phages (35). The mechanism by which LydB prevents premature cell lysis is unknown. No significant homology was found for lydB and the lydB gene product in a search of the GenBank/EMBL and SwissProt databases, respectively.

Mapping, cloning, and sequence analysis of gene 17. Marker rescue experiments with the gene 17-deficient amber mutant am17.20 by Citron et al. (6) located gene 17 on a 0.9-kb KpnI-HaeIII restriction fragment at map position 21 (33). This KpnI-HaeIII restriction fragment (which is identical to the KpnI-BalI fragment; see below) had been cloned on a multicopy plasmid, resulting in plasmid pUM4.1 (Fig. 1). Cells harboring pUM4.1 were found to complement the mutant phenotype of am17.20. This result suggested that gene 17 and its promoter sequence are contained within the KpnI-HaeIII restriction fragment of pUM4.1 (6).

The single KpnI and BsaBI restriction sites of pUM4.1 were utilized for the construction of a set of nested deletions by using exonuclease III and S1 nuclease. The resulting subclones served directly as templates in the double-stranded sequencing reactions and facilitated the determination of the 871-bp nucleotide sequence of the insert carried in pUM4.1 (Fig. 1). Cells harboring pUM4.1 were found to complement the mutant phenotype of am17.20. This result suggested that gene 17 and its promoter sequence are contained within the KpnI-HaeIII restriction fragment of pUM4.1 (6).

To test whether this open reading frame corresponds to gene 17, a 0.9-kb fragment including late promoter LPr21, gene 17, and the nucleotide sequence downstream of gene 17 up to the BalI restriction site was amplified from an am17.20 phage lysate by PCR. The product of this PCR amplification served directly as template for the sequencing reactions. A single base change at position 377 compared with the wild-type sequence was found. The G-to-A transition mutated a tryptophan codon
indicated by asterisks. The two arrowson the rightsid eof the figure indicate the strandsof the initiation sites shownat the left side of the figure, withthenucleotides of the initiation sites indicated by asterisks. The two arrows on the right side of the figure indicate the two primer extension products.

(TGG) to an amber stop codon (TAG). This result confirmed that gene 17 is identical to the 558-bp open reading frame present on plasmid pUM4.1.

Mapping of the LPr21 late promoter sequence. In vitro primer extension studies by Citron et al. (6) with RNA polymerase holoenzyme Er70 revealed a repressor-controlled promoter sequence, Pr21, but no signal was found on the complementary strand where gene 17 is encoded. Expression from the late phage-specific promoter sequence, LPr21, which requires activation by gp10, was now mapped by primer extension analysis (Fig. 4) in an induced P1 lysogenic strain harboring plasmid pAW1438. Plasmid pAW1438 carries the 195-bp KpnI–ApoI restriction fragment with the LPr21 late promoter sequence on a multicopy plasmid (Fig. 1). Two signals at two consecutive T nucleotides were identified as transcription initiation sites (Fig. 3), confirming the location and functionality of late promoter sequence LPr21.

Construction of a lysis cassette. To study the antagonist effect of LydB on LydA, an in vivo system was designed. The 871-bp KpnI–BalI restriction fragment harboring gene 17 and the 0.9-kb AgeI restriction fragment (from the AgeI site at position 1 to an AgeI site located approximately 50 bp downstream of the SexAI site) harboring hydA and hydB were cloned on low-copy-number plasmid pWSK30 under the control of the inducible T7 late promoter sequence, resulting in plasmid pAW1440 (Fig. 1). The three genes are arranged as a polycistron, thus differing from the regulon organization on the P1 chromosome, where hydA and hydB are cotranscribed from late promoter sequence LPdar and gene 17 from LPr21, with both promoters being activated by gp10. Plasmid pAW1442 differs from pAW1440 in that hydB has been deleted.

The optical density at 600 nm of the three strains harboring pAW1442, pAW1440, or the pWSK30 plasmid and plasmid pGP1-2 with the heat-inducible T7 RNA polymerase was monitored with and without induction (Fig. 5). Lysis of the strain harboring pAW1442, which expresses gene 17 and hydA, is seen between 15 and 30 min after induction and is indicated by a strong decrease in optical density. Compared with the strain harboring vector pWSK30, the strain harboring pAW1440 (in which hydB is also expressed) experiences only a small decrease in optical density. However, after the addition of chloroform, the strain harboring pAW1440 lysed efficiently.

Samples from induced cells harboring one of the two lysis cassettes as well as from induced P1 lysogenic cells were examined by light microscopy (data not shown). Approximately 40 min after induction, lysogenic cells formed spheres for a few seconds before they collapsed. Almost all cells expressing LydA and gp17 formed spheres 15 min after induction. Surprisingly, these cells remained spherical and did not collapse. Only a few cells expressing LydA, LydB, and gp17 formed spheres 60 min after induction. However, addition of chloroform to both cultures expressing one of the lysis cassettes resulted in the disruption of all cells (spherical and nonspherical). The striking difference in lysis behaviors in the absence and presence of LydB confirms the expectation that LydB acts as an antagonist of LydA.

**DISCUSSION**

Lysis of the host cell by many types of bacteriophages was found to depend on at least two different sets of proteins (for a review, see reference 35). These proteins are grouped according to the structures of their targets in the cell. Degradation of the cell wall is exerted by endolysins, e.g., the T4 e gene product, a lysozyme, or the λ R protein, a transglycosylase. Endolysins identified so far do not have a signal sequence and require the action of a membrane-disrupting protein in order to reach the peptidoglycan of the cell wall. The other group of proteins are the holins, also known as the hole former in the plasma membrane (e.g., the λ S gene products S105 and S107).
In most cases, two nearly identical proteins are used for adequate timing of cell lysis. Because of the so-called dual-start motif (Met-Lys-Met for the λ S gene), two proteins are expressed from a single gene differing only in a short positively charged extension at the N terminus (the dipeptide Met-Lys for the λ S gene). Premature cell lysis occurs when only the shorter protein (S105) is expressed in the infected cell (2). The S107 protein is thought to be an antagonist of premature lysis by S105 (4).

In this study, three proteins of bacteriophage P1 involved in cell lysis were identified. Homologies of LydA and gp17 to other phage lysis proteins were found. LydA, in the amino-terminal part, shares with the phage holins two putative membrane-spanning domains separated by a β-turn, whereas homology of the deduced amino acid sequence of gene 17 to several proteins of the T4 lysozyme family was found (Fig. 6). The highest similarity of gp17 was found with an unassigned open reading frame in the Haemophilus influenzae bacteriophage HP1 (Hp1.Hi), the R2 gene product of the lambdoid phage 21 (21.Ec), gp19 of the S. typhimurium phage P22 (P22.St), gp15 of the B. subtilis phage f29 (f29.Bs); the C-terminal 75 residues are omitted, the lysin gene product of the L. lactis phage dvML3 (dvML3.Ll; the C-terminal 49 residues are omitted), and the e gene product of phage T4 (T4.Ec). Residues similar for at least four of seven sequences for a particular position are boxed. The three conserved regions I to III are indicated by a solid line above the P1gp17 sequence. The glutamate residue of the active center in the T4 lysozyme is marked with an arrow above the P1gp17 sequence (see text for references).

The function of the third protein, which showed no homology with other phage lysis proteins or with proteins in the SwissProt database, was studied in an in vivo system expressing gene 17, lydA, and lydB together or gene 17 and lydA (no lydB) from the inducible T7 promoter sequence. A delay of cell lysis expressing LydB revealed an antagonist effect of LydB on LydA. This result is consistent with the phenotype of the lydB mutant phage am2.3.1, which was found to prematurely lyse its host cell. In an analogy to the phage λ holins, LydA exerts the function of S105 and LydB exerts the one of S107. However, P1 uses a novel mechanism to control the time point of cell lysis. The antagonist LydB is genetically distinct from LydA. Indeed, no homology of LydB with LydA or any potential transmembrane domain in LydB has been identified. LydB might therefore block the holes formed by LydA upon binding to the cytoplasmic domain of these holes. Alternatively, LydB might affect the stability of LydA or the lydA transcript, thereby regulating the number of hole-forming complexes.

Regulation of the time point of lysis has been reported to occur also during bacteriophage P2 lytic infection, in which two nonessential genes, lysA and lysB, are involved (37). However, their gene products have only a minor effect, resulting in a 2- to 5-min acceleration or delay of lysis when lysA or lysB, respectively, is deleted. No homology of lysA or lysB with each other or with the P1 lydB gene has been identified.

There is evidence for a fourth lysis function in bacteriophage P1. No complete complementation of P1vir am17.20 with the KpnI-BalI restriction fragment on multicopy plasmid pUM4.1 was found in a marker rescue experiment by Citron et al. (6), suggesting that this amber mutation has a polar effect. However, full complementation was found with the BamHI-A1 restriction fragment on medium-copy-number plasmid pMC4.1. This plasmid contains an additional 2 kb downstream of gene 17. Sequence analysis downstream of the BalI site revealed an additional open reading frame (data not shown) which might be a functional analog to the λ R0 gene product, a putative auxiliary endolysin, although no homologies were identified. Absence of this fourth lysis gene from the lysing cassette may be the cause of the stable spherical cells that were observed under light microscopy and that were similar to the ones which appeared after infection with the λ R0 mutant phage (36).

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**FIG. 6. Alignment of the amino acid sequences of bacteriophage P1 gp17 (P1.Ec), the product of an unassigned open reading frame of the H. influenzae phage HP1 (Hp1.Hi), the R2 gene product of the lambdoid phage 21 (21.Ec), gp19 of the S. typhimurium phage P22 (P22.St), gp15 of the B. subtilis phage f29 (f29.Bs); the C-terminal 75 residues are omitted, the lysin gene product of the L. lactis phage dvML3 (dvML3.Ll; the C-terminal 49 residues are omitted), and the e gene product of phage T4 (T4.Ec).**

Residues similar for at least four of seven sequences for a particular position are boxed. The three conserved regions I to III are indicated by a solid line above the P1gp17 sequence. The glutamate residue of the active center in the T4 lysozyme is marked with an arrow above the P1gp17 sequence (see text for references).
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