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bldA-Dependent Expression of the *Streptomyces exfoliatus* M11 Lipase Gene (*lipA*) Is Mediated by the Product of a Contiguous Gene, *lipR*, Encoding a Putative Transcriptional Activator

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Extracellular lipase synthesis by *Streptomyces lividans* 66 carrying the cloned lipase gene (*lipA*) from *Streptomyces exfoliatus* M11 was found to be growth phase dependent, since lipase was secreted into the medium mainly during the stationary phase; S1 nuclease protection experiments revealed abundant *lipA* transcripts in RNA preparations obtained during the stationary phase but not in those obtained during exponential growth. Transcription from the *lipA* promoter was dependent on the presence of *lipR*, a contiguous downstream gene with a very high guanine-plus-cytosine content (80.2%). The deduced *lipR* product consists of a protein of 934 amino acids that shows similarity to known transcriptional activators and has a strong helix-turn-helix motif at its C terminus; this motif is part of a domain homologous to DNA-binding domains of bacterial regulators of the UhpA/LuxR superfamily. The *lipR* sequence revealed the presence of a leucine residue, encoded by the rare TTA codon, which caused *bldA* dependence of *lipA* transcription in *Streptomyces coelicolor* A3(2); replacement of the TTA codon by the alternate CTC leucine codon alleviated *bldA* dependence but not the apparent growth phase-dependent regulation of *lipA* transcription. When *lipR* expression was induced in a controlled fashion during the exponential growth phase, by placing it under the inducible *tipA* promoter, lipase synthesis was shifted to the exponential growth phase, indicating that the timing of *lipR* expression, and not its *bldA* dependence, is the main cause for stationary-phase transcription of *lipA*.

Members of the genus *Streptomyces* are gram-positive soil eubacteria characterized by a mycelial growth habit. Their complex life cycle involves both morphological and physiological differentiation (5). Streptomycetes are endowed with a remarkable capacity for synthesis of secondary metabolites, which are usually produced in a growth phase-dependent manner; in liquid batch culture, synthesis of antibiotics and other secondary metabolites usually starts at the onset of the stationary growth phase (reviewed in reference 3).

Several gene products play an important role in the morphological and physiological differentiation process (5); among these, the product of the *bldA* gene has been shown to be essential for morphological differentiation, and in addition it affects the synthesis of many secondary metabolites (24, 25). The *bldA* gene encodes the only tRNA capable of efficiently translating the leucine codon TTA (26), which is rarely present in streptomycete genes and appears to be absent from genes encoding essential proteins, since the *bldA*-encoded tRNA is dispensable for vegetative growth (25). TTA codons have been found, however, in genes encoding proteins involved in regulation of antibiotic production and morphological differentiation (25).

Lipases (triacylglycerol acylhydrolases [EC 3.1.1.3]) are an important group of enzymes with many industrial applications (45). A survey of lipase-producing microorganisms from soil showed that members of the genus *Streptomyces* can be highly lipolytic (42), which prompted us to initiate the study of these enzymes at the molecular level. We have previously described the cloning of lipase-encoding genes from *Streptomyces exfoliatus* M11 (formerly *Streptomyces* sp. strain M11) and *Streptomyces albus* G and shown that similar genes might be commonly found among *Streptomyces* species (6, 34). In addition, we have shown that these *Streptomyces* lipases are similar to one of the three lipases (lipase 1) of the psychrotroph *Moraxella* sp. strain TA144 (8); the similarity of bovine isoform II of platelet-activating factor acetylhydrolase to the *Streptomyces* lipases has also recently been described (14). Previous observations suggested that an accessory gene was necessary to achieve a high level of expression of the cloned *S. exfoliatus* M11 lipase gene in *Streptomyces lividans*, since cloning the structural gene alone in the absence of downstream sequences present in the insert of the original clone resulted in a much smaller amount of lipase being secreted into the medium (34).

In this paper we report that *bldA*-dependent expression of the *S. exfoliatus* M11 lipase gene, *lipA*, is mediated by the product of a contiguous gene, *lipR*, which encodes a putative transcriptional activator.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and microbiological procedures. The *Escherichia coli* K-12 strain used throughout this study was JM101 (49), which was grown under standard conditions (29); transformation of *E. coli* with plasmid DNA was carried out as described by Inoue et al. (17). Wild-type *S. lividans* 66 was used throughout this study (strain 1326 from the John Innes Institute collection); the *Streptomyces coelicolor* A3(2) strains used were J1501 (*hisA1 uraA1 strA1 pgl* SCP1$^-$ SCP2$^-$) and its isogenic *bldA39* derivative, J1700 (kindly provided by K. F. Chater). Transformation of *Streptomyces* strains with plasmid DNA was carried out as described by Hopwood et al. (16) except that protoplasts were plated in a hypertonic soft agar overlay (28). The plasmids used are listed in Table 1.

DNA manipulation and sequence analysis. Subcloning of DNA fragments was done by standard procedures (36). Nucleotide sequencing of both strands of the pB13 insert was done as described previously (34). Sequence assembly and analysis were carried out with PC/GENE release 6.85 from Intelligenetics; in

TABLE 1. Plasmids used in this study

Plasmid	Description and derivation	Reference
pB13	Original lipase clone obtained by insertion of partial *Sau*3A digests of *S. exfoliatus* M11 DNA in the *Bam*HI site of pIJ486	34
pB28	Insert from pB13 cloned as a *Bgl*II fragment in the single *Bam*HI site of pIJ903	This work
pB36	Entire insert from pB13 taken out as an *Xba*I-*Sst*I fragment, blunt-ended with Klenow, and cloned into *Sma*I-cut pIJ2925	This work
pB47	Obtained by religating *Bst*EII-*Xba*I-cut pB36 after blunt-ending with Klenow. Carries only *lipA* with associated promoter region	This work
pB48	Mel$^+$ Hyg^r vector derived from the pJV1 minimal replicon	39
pB49	2.1-kb *Apa*I fragment carrying a promoterless *lipA* gene cloned into the *Sma*I site of the pIJ2925 polylinker; transferred as a *Bgl*II fragment into pIJ702 followed by insertion of a *Sst*I fragment carrying *dagAp*$_4$ and *dagAp*$_3$ promoters in front of *lipA*	This work
pB51	Insert from pB47 cloned as an *Eco*RI-*Hin*dIII fragment in pIJ486. Carries only *lipA* with its associated promoter region	This work
pB72	Derivative of pIJ2925 with modified polylinker from pUCBM21 (Boehringer Mannheim)	38
pB94	Derivative of pIJ486 with *lipA* and *lipR* genes but not *orf481*. The TTA codon inside *lipR* has been replaced by a CTC codon. Same orientation as in pB13	This work
pB96	Same as pB94 but retains the TTA codon inside *lipR*	This work
pB98	Derivative of pB72 carrying the 364-bp *Hin*dIII-*Sst*II fragment of pB13, containing part of the pIJ486 polylinker, the *lipA* promoter region, and the N-terminal part of *lipA* up to the first *Sst*II site	This work
pB99	Derivative of pIJ6021 with *lipR* cloned under the control of the *tipA* promoter	This work
pB100	Insert from pB47 cloned as a *Bgl*II-*Pst*I fragment into pB48	This work
pB104	Derivative of pIJ6021 with *lipA* cloned under the control of the *tipA* promoter	This work
pIJ486	High-copy-number *Streptomyces* cloning vector, derived from the pIJ101 replicon	46
pIJ702	High-copy-number *Streptomyces* cloning vector, derived from the pIJ101 replicon	20
pIJ903	*E. coli*-*Streptomyces* shuttle vector derived from the low-copy-number SCP2* replicon	27
pIJ2925	Derivative of pUC18 with modified polylinker flanked by *Bgl*II sites	19
pIJ6021	Kan^r high-copy-number inducible expression vector for *Streptomyces*, derived from the pIJ101 replicon and carrying the strong and inducible *tipA* promoter	44

particular, the programs ASSEMGEL, PCOMPARE, PALIGN, REGULAT, and CLUSTAL were used. Database searches were done with the BLASTP program (1). In order to subclone the *lipA* and *lipR* genes under the control of the *tipA* promoter region present in the inducible expression vector pIJ6021 (44), *Nde*I sites were introduced at their respective translational start codons; to change the TTA codon in the *lipR* sequence, it was replaced by the alternate leucine codon CTC, thereby generating an *Sst*I site. These sites were introduced either by oligonucleotide-directed mutagenesis according to the method of Kunkel (23) with a U-DNA mutagenesis kit from Boehringer Mannheim or by PCR with AmpliTaq DNA polymerase from Perkin-Elmer. All mutations were confirmed by DNA sequencing.

Growth of cultures and assay of lipase activity. Luria-Bertani medium (29) supplemented with 34% sucrose and 50 ppm of antifoam A (Sigma) was used. Cultures (30 ml) were grown at 30°C in 250-ml siliconized flasks with stainless steel springs (16) at 250 rpm in an orbital shaker. Thiostrepton was added at 20 μg ml^{-1} for plasmid maintenance in liquid cultures and at 5 μg ml^{-1} to induce the *tipA* promoter; hygromycin B was added at 50 μg ml^{-1} and kanamycin was added at 100 μg ml^{-1}. Cultures were inoculated with spores washed twice in sterile distilled water, except for the *bldA39* strain, J1700, and its wild-type parent, J1501, which were inoculated with small mycelial fragments pregrown in the same medium as described previously (28). Growth was monitored by optical density at 450 nm. Samples to be assayed for lipase activity were drawn at appropriate time intervals and centrifuged at 16,000 $\times$ *g* in a microcentrifuge; the supernatant was carefully transferred to a fresh tube and stored at 4°C until it was assayed. Lipase activity was determined by a colorimetric esterase assay with *p*-nitrophenyl-laurate as the substrate, as previously described (34). One lipase unit is defined as the amount of enzyme which liberates 1 μmol of *p*-nitrophenol per min, considering an extinction coefficient of 14,775 (35).

Lipase production was also assayed qualitatively by the formation of fluorescent halos on olive oil-rhodamine B plates (22) as previously described (34).

Production of anti-lipase antibodies. A polyacrylamide gel slice containing purified lipase (34) was homogenized in phosphate-buffered saline solution, mixed with an identical volume of Freund's complete adjuvant, and injected subcutaneously into a New Zealand White rabbit (10 μg in 0.5 ml). Two booster immunizations were given at 3-week intervals. Following the immunizations, the rabbit was bled and serum was prepared from the whole blood.

Protein electrophoresis and Western blot analysis. Total protein in 300-μl samples of culture supernatants was precipitated by first adding NaCl to 0.2 M followed by the addition of trichloroacetic acid to a final concentration of 15% and incubating the samples on ice for 1 h; the samples were then centrifuged, and the protein pellet was washed twice with a cold acetone-ethanol mixture and dried. The precipitated protein was then resuspended in 50 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and 10-μl samples were run directly on gels after denaturation at 90°C for 5 min. SDS-PAGE was carried out with a 4% stacking gel and a 10% separating gel in a Mighty Small II electrophoresis unit (Hoefer Scientific Instruments) according to the instructions of the manufacturer. Proteins were electrotransferred to Hybond nitrocellulose (Amersham) for Western blot analysis, which was performed by standard procedures (12a) with a 1:50 dilution of the rabbit antiserum followed by goat anti-rabbit immunoglobulin G–horseradish peroxidase conjugate (GIBCO-BRL) and visualization of the complexes with 4-chloro-1-naphthol.

S1 nuclease protection assays. Total RNA isolation from *Streptomyces* cultures was done according to the method of Hopwood et al. (16). For probe preparation, pB98 (Table 1) was cut with *Sun*I, dephosphorylated, and labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase; a second cut was carried out with *Pst*I, and the 320-bp fragment was purified from a low-melting-point agarose gel. Approximately 50 ng of this fragment was hybridized to 50 μg of total RNA in 20 μl of 3 M sodium trichloroacetate (NaTCA) hybridization buffer (31) for 5 h; NaTCA was prepared as described previously (41). Further processing of the samples and denaturing gel electrophoresis of the protected fragments were done as previously described (37). End-labelled pBR322-*Hpa*II fragments were used as size markers. The expected size of fragments protected by *lipA* transcripts is 167 to 169 nucleotides (nt) (34).

RESULTS

The cloned *S. exfoliatus* M11 *lipA* gene directs growth phase-dependent lipase synthesis in *S. lividans*. We have previously reported that when *S. lividans* 1326 carrying the cloned extracellular lipase gene (*lipA*) from *S. exfoliatus* M11 in a high-copy-number plasmid (pB13 [Table 1]) is grown in rich Luria-Bertani medium, lipase accumulation is observed and large amounts of lipase protein can be recovered from the culture supernatant (34). When the timing of lipase synthesis by these cultures was analyzed more carefully, we found that most of the lipase activity appeared in the medium once the culture had entered stationary phase. As can be seen in Fig. 1, very little lipase activity was found in the medium during exponential growth but lipase activity increased constantly once the cultures entered stationary phase. Gel electrophoresis of total protein from 1326(pB13) culture supernatants (Fig. 2A) re-

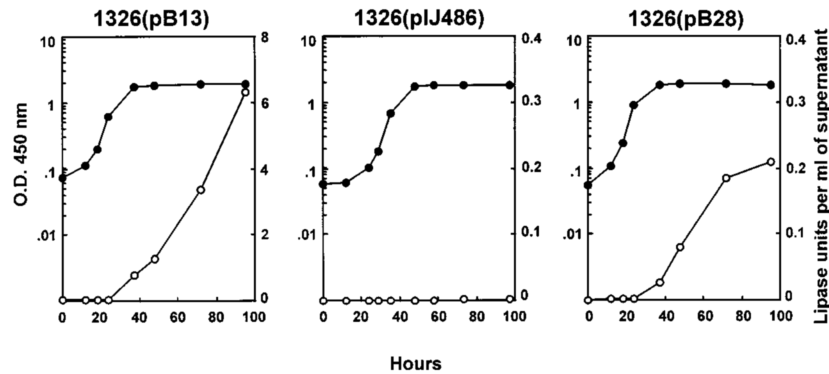

FIG. 1. Growth of *S. lividans* 1326 carrying different plasmids (●) and lipase activity in the supernatant (○). Plasmid pB13 is one of the original clones described previously (34) that has a 6.1-kb insert of *S. exfoliatus* M11 DNA cloned into the pIJ101-derived vector pIJ486 (46). Plasmid pB28 carries the same insert cloned in the SCP2*-derived vector pIJ903 (27). O.D., optical density.

vealed no significant amounts of extracellular proteins by 24 h, which usually corresponded to early exponential phase. By 48 h, which corresponded to early stationary phase, most proteins in the supernatant had been secreted, including a polypeptide with an apparent molecular mass of 30 kDa. No significant increase in the amounts of most proteins, and in fact a decrease in some, could be observed at 72 and 96 h, with the exception of the 30-kDa polypeptide, which continued to be secreted during the stationary phase; this protein showed a mobility identical to that of the purified lipase. A Western

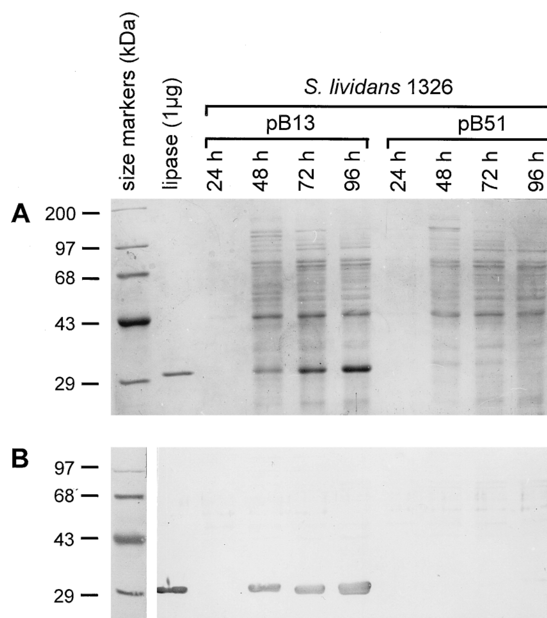

FIG. 2. Analysis of extracellular proteins produced by *S. lividans* 1326 carrying different plasmids. Plasmid pB13 carries the 6.1-kb insert including the *lipA* gene and promoter and additional downstream DNA, whereas pB51 carries only *lipA* and its associated promoter with no additional downstream DNA (Table 1). (A) SDS-PAGE of proteins in culture supernatants stained with Coomassie brilliant blue R-250. The times at which the fractions were taken from the cultures, as well as the cultures from which they originate, are indicated above the lanes. The molecular mass, in kDa, of the size markers is indicated at the left. Pure lipase was obtained as described previously (34). (B) Western blot of a gel identical to that shown in panel A with rabbit anti-lipase polyclonal antibodies. After transfer to nitrocellulose, the lane containing the size markers was cut from the filter, stained with a 0.2% solution of red Ponceau in 3% acetic acid, and saved for comparison.

blotting experiment with anti-lipase polyclonal antibodies showed that the 30-kDa polypeptide, which accumulated during the stationary phase in 1326(pB13) supernatants, indeed corresponded to the *lipA*-encoded lipase (Fig. 2B); cultures of *S. lividans* 1326 carrying the vector pIJ486 did not show the presence of this polypeptide at any point of the growth curve (34 and data not shown). Therefore, lipase activity in the culture supernatants provided a reliable indication of the amount of lipase synthesized and secreted into the medium, and it was subsequently used to assay the amount of lipase produced by different cultures. Reliable determination of lipase activity was greatly facilitated by the stability of the *lipA*-encoded lipase: less than a 10% loss in activity was observed after incubation of crude culture supernatants at 30°C for up to 1 week, and no significant loss was seen at 4°C after several weeks.

Since the observed increase in lipase secretion after cessation of growth could merely reflect an increase in plasmid copy numbers after the cultures entered stationary phase, the insert from pB13 was transferred as a *Bgl*II fragment into the *Bam*HI site of pIJ903, an SCP2*-derived vector whose copy number is tightly controlled and maintained at one to two copies per chromosome (3a, 27). When lipase secretion into the medium by *S. lividans* 1326 carrying this plasmid (pB28) was measured, a pattern of lipase accumulation similar to that of 1326(pB13) was observed: while maximal lipase activity in the medium was about 20 to 30 times lower than that obtained with 1326(pB13) cultures (reflecting the reduced presence of *lipA*), most of the lipase activity appeared after the cultures entered stationary phase. This result suggested that lipase secretion by cultures of *S. lividans* carrying the cloned *lipA* gene was growth phase-dependent and mostly occurred after exponential growth had ceased.

Lipase gene transcription requires the product of an additional gene. Only very small amounts of lipase activity were found in the medium when 1326(pB51) cultures were analyzed (Fig. 3); pB51 carries the *lipA* gene and promoter but lacks any sequences downstream of *lipA* that are present in the original pB13 insert. In addition, gel electrophoresis and Western blot analysis of total protein in the supernatant of 1326(pB51) cultures failed to reveal a significant amount of lipase polypeptide (Fig. 2). This confirmed our previous observation that *lipA* expression requires additional DNA sequences present in pB13 (34). This requirement could be at the transcriptional level, if *lipA* could only be transcribed from its own promoter in the presence of some additional factor encoded in pB13, or at the posttranscriptional level if additional factors were re-

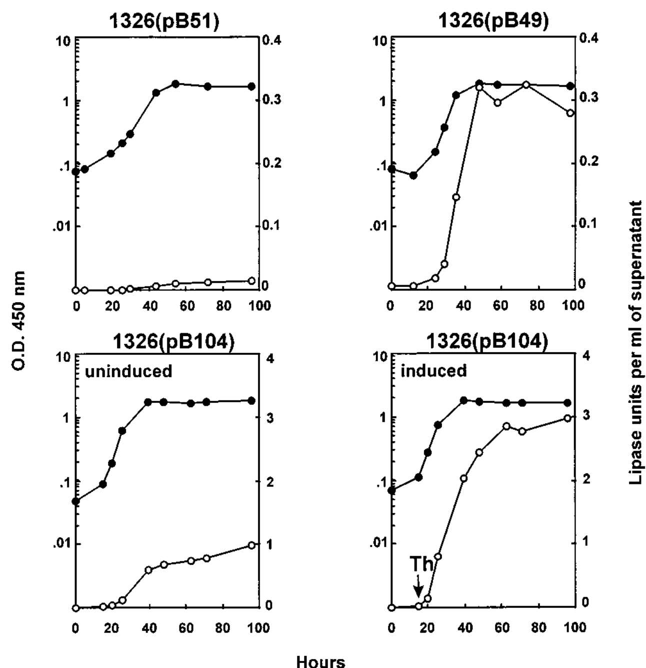

FIG. 3. Growth of *S. lividans* 1326 carrying different plasmids (●) and lipase activity in the supernatant (○). Plasmid pB51 carries the *lipA* gene and promoter but lacks any additional downstream sequences that were present in the original insert, pB49 carries the promoterless *lipA* fused to the $dagAp_4$ and $dagAp_3$ promoters, and pB104 carries the *lipA* gene under the transcriptional and translational control of the *tipA* promoter region. Induction of the *tipA* promoter was achieved by adding 5 μg of thiostrepton (Th) ml^{-1} at the time indicated by an arrow. O.D., optical density.

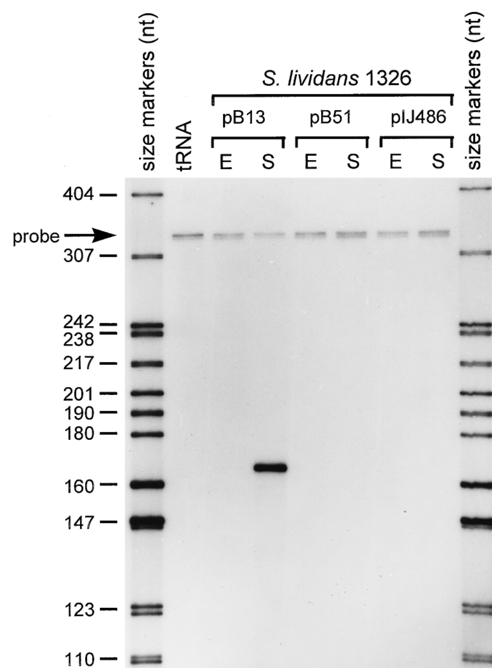

FIG. 4. Transcription of *lipA* in *S. lividans* 1326 cultures carrying different plasmids. Total RNA was isolated from cultures during the exponential growth phase (E; optical density at 450 nm readings between 0.45 and 0.9) or after entry into the stationary phase (S). The length in nucleotides of size markers is indicated to the left. Fragments protected by *lipA* transcripts should be 167 to 169 nt long. A small amount of reannealed full-length probe (320 nt [arrow]) can be seen in all lanes, including a control hybridization with 50 μg of tRNA.

quired for translation of the *lipA* message or for lipase processing and/or secretion. In order to determine which was the case, the *lipA* gene was placed under the control of the regulatory regions of other streptomycete genes. In particular, *lipA* was placed under the transcriptional control of the $dagAp_4$ and $dagAp_3$ promoters (4) in pB49 and under the transcriptional and translational control of the *tipA* regulatory region (15, 30) in pB104. These different plasmids were introduced into *S. lividans* 1326, and the temporal pattern of lipase secretion into the medium was analyzed. As can be seen in Fig. 3, lipase secretion into the medium occurred in all cases. In addition, it is possible to see that these constructs differ both in the amount of lipase secreted into the medium and in the temporal pattern of expression. In 1326(pB49) cultures lipase secretion into the medium coincided with the exponential growth phase and no additional increase was seen once the cultures entered the stationary phase; the amount of lipase secreted, however, was lower than that observed when *lipA* was transcribed from its own promoter in pB13. Higher lipase levels, similar to those found in 1326(pB13) cultures, were obtained in 1326(pB104) cultures, in which *lipA* was fused to the strong and inducible *tipA* promoter (Fig. 3). When transcription from the *tipA* promoter was induced with thiostrepton at the start of the exponential growth phase, lipase production started as soon as the thiostrepton was added and large amounts of lipase were secreted into the medium during the exponential growth phase; lipase accumulation continued during the stationary phase, although at a much lower rate. These results show that mycelium growing exponentially is capable of synthesizing and secreting high levels of lipase into the medium in the absence of any additional factors encoded in pB13 but only when *lipA* is transcribed under the control of other promoters ($dagAp_4p_3$ or *tipA*) which are expressed during the exponential growth phase. Therefore, the most likely explanation for the lack of lipase synthesis by 1326(pB51) cultures is that *lipA* transcription from its own promoter requires an additional factor encoded in pB13. To confirm this, total RNA was purified from cultures of *S. lividans* 1326 carrying either pB13, pB51, or the vector pIJ486, and the amount of *lipA* transcripts in these RNA preparations was determined by S1 nuclease protection experiments (Fig. 4). The results show a significant number of *lipA* transcripts in RNA preparations from 1326(pB13) cultures, whereas no transcripts could be detected in 1326(pB51) RNA preparations. Interestingly, *lipA* transcripts could only be detected in RNA preparations of 1326(pB13) cultures obtained during the stationary phase. Therefore, *lipA* transcription requires some factor encoded in pB13 and appears to be growth phase dependent.

Sequence of the pB13 insert reveals a gene encoding a potential transcriptional activator. To identify any factor necessary for efficient *lipA* transcription, the sequence of the entire pB13 insert was obtained (Fig. 5). Analysis of the DNA sequence revealed the presence of two downstream open reading frames (ORFs) in addition to *lipA* (Fig. 6); both of these ORFs would be transcribed in the same orientation as *lipA*. The start of the first ORF (*orf934*, encoding a protein of 934 amino acids) had already been observed when the *lipA* sequence was reported (34); it starts 399 bp downstream of *lipA* and ends just before the rightmost *Kpn*I site of the insert (at nt 4450 [Fig. 6]). The second ORF (*orf481*, encoding a protein of 481 amino acids) starts just downstream of the same *Kpn*I site and ends a few nucleotides upstream of the right-hand end of the pB13 insert (Fig. 6). In order to find whether the product of either

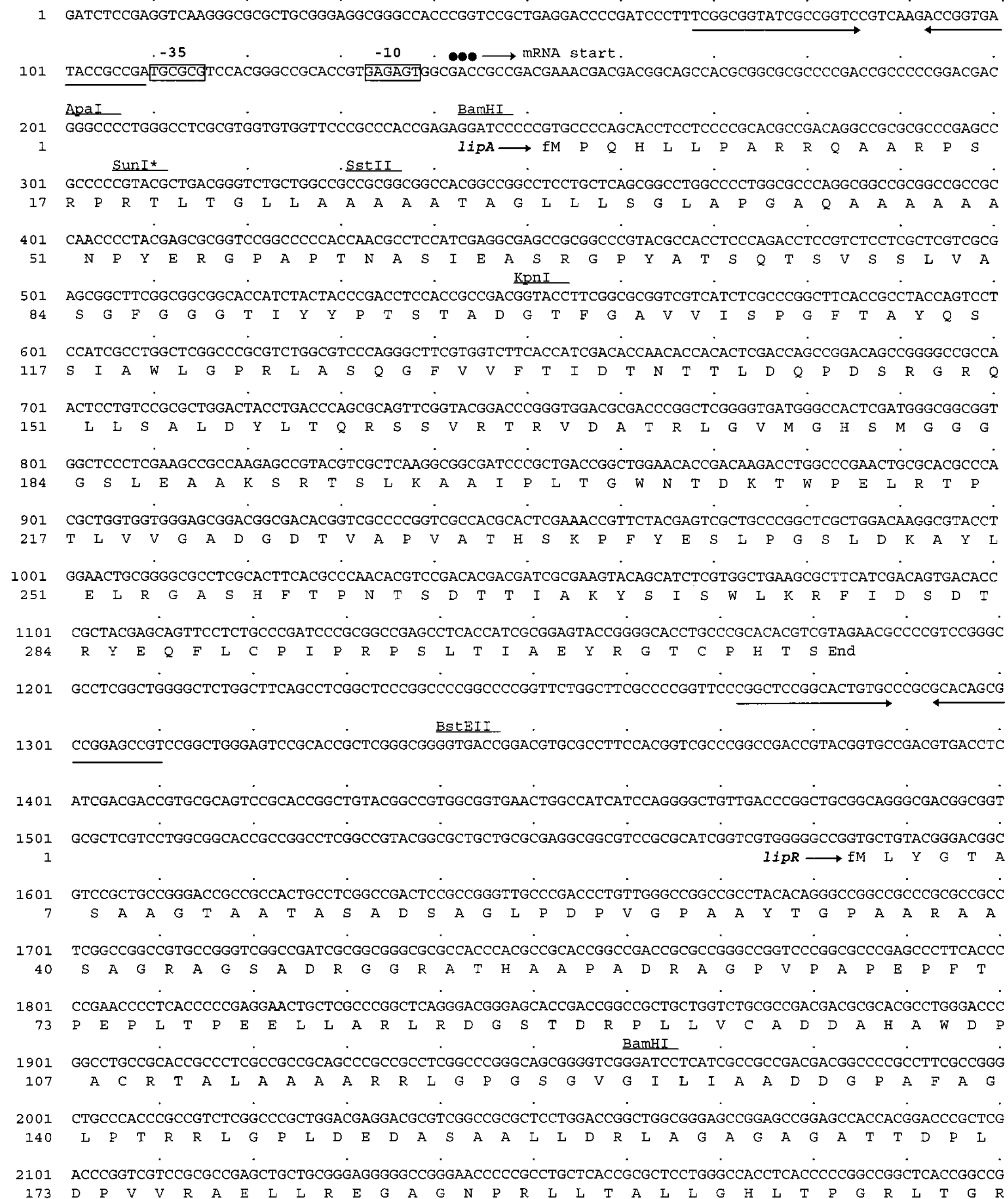

FIG. 5. Nucleotide sequence of the entire pB13 insert. The −35 and −10 regions of the *lipA* promoter (34) are boxed. The start of the *lipA* mRNA is shown as three bullets followed by an arrow. Relevant restriction sites are shown above the sequence. The *Sun*I site used for S1 nuclease protection experiments is marked with an asterisk. Not all *Apa*I, *Sst*II, or *Sun*I sites are shown. Inverted repeats are indicated by arrows below the sequence. The TTA codon inside the *lipR* sequence is in reverse type, and the helix-turn-helix motif in LipR is boxed and in bold letters. The GenBank accession number for this sequence is M86351.

one or both of these ORFs was required for *lipA* transcription, a series of deletions were obtained with the help of exonuclease III, starting at the right-hand end of the pB13 insert; plasmids carrying these deletions were introduced by transformation into *S. lividans* 1326, and lipase production by these cultures was quantified and compared to that of 1326(pB13) and 1326(pB51) cultures. *S. lividans* 1326 carrying plasmids with deletion endpoints located to the right of the *Kpn*I site at nt 4450 (which removed most of *orf481*) secreted lipase at about the same levels as cultures carrying pB13, whereas cultures carrying plasmids with deletion endpoints to the left of the same *Kpn*I site (which removed the C-terminal part of *orf934*) showed a dramatic decrease in lipase synthesis, comparable to that observed for 1326(pB51) cultures (Fig. 6). Therefore, only the product of *orf934* appeared to be required for *lipA* transcription, and it was accordingly renamed *lipR.*

Analysis of the *lipR* sequence revealed an extremely high guanine-plus-cytosine (G+C) content: an average of 80.2 mol% throughout the length of the gene, which is significantly higher than the average 74 mol% of most *Streptomyces* genes

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                                                                                          ApaI
2201 CACCCCGCTGCCCTCTCCGCTGCCCGGGGCCGAAGGGGTGCTGGAGGCGTACGCGTACCGCCTGGACGCCCTCCCCGCGCCCGCCCGGGCCCTGCTCCTG
 207   T  P  L  P  S  P  L  P  G  A  E  G  V  L  E  A  Y  A  Y  R  L  D  A  L  P  A  P  A  R  A  L  L  L

2301 CTGGCCGCCGCGGCCCAGGAGCACGAGCCCGCCGGGGCGGGTACGGACGCGCTGCTCCTGCTGCGCGCGGGCACCCGGGCCGGGCTGCCCCGGGACTTCC
 240  L  A  A  A  A  Q  E  H  E  P  A  G  A  G  T  D  A  L  L  L  L  R  A  G  T  R  A  G  L  P  R  D  F

2401 TGGACGACGCCCTGTTCGGTCCGGCCGCGACCGAGGGGCTGCTGCAACGGGCGGGGAGCCGGGTCCACTTCAGCCCGCCCCTGGCCGCCCGAGCGGTCCT
 273 L  D  D  A  L  F  G  P  A  A  T  E  G  L  L  Q  R  A  G  S  R  V  H  F  S  P  P  L  A  A  R  A  V  L

2501 GCACCACACGCCCCCGGCCCGCCGCCGCGCCGCCCACGAACTGCTCGCCGCGCTGCTGGGGGAGGCGGGCGGCCGGGCCGGAACGCTCGCCGCCCTCGCC
 307   H  H  T  P  P  A  R  R  R  A  A  H  E  L  L  A  A  L  L  G  E  A  G  G  R  A  G  T  L  A  A  L  A

2601 CAGCGGGCCTGTGCCGCGCCGGGCCCCGACCACGCGCTCGCCGCCCGGCTGGAGGCGGCGGCCACCGCCCCGTACTCCCACGCCGAACGCTCCTCCGCCC
 340  Q  R  A  C  A  A  P  G  P  D  H  A  L  A  A  R  L  E  A  A  A  T  A  P  Y  S  H  A  E  R  S  S  A

2701 TGGCCCGCGCCGCCGCGCTCACCCCCGATCCGCCGCTGCGCTCGGCCCGGTTCGCCGCAGCCGCCGAACAGGCGGCCCACGCGGGCGACCCGGACCGGGC
 373 L  A  R  A  A  A  L  T  P  D  P  P  L  R  S  A  R  F  A  A  A  A  E  Q  A  A  H  A  G  D  P  D  R  A

2801 CCGCGCCCTCCTGGCCCGCACGGCCGCCCCGGCGGAAACGGGGTCCGGCCTCGCGCCTACGACGGGTGGGCCGGGGCTCGCCCCCTACGTCCACGGCCTG
 407   R  A  L  L  A  R  T  A  A  P  A  E  T  G  S  G  L  A  P  T  T  G  G  P  G  L  A  P  Y  V  H  G  L
                            SalI
2901 CTCGCCCTGCGTTCCGGCCCCGCCGTCGACGCCCACGAAGCTCTCCTGGCCGCCGCAGCGCTGCTCGGGCCGCACGATTCCGGCCGGGCCCTCCACGCGC
 440  L  A  L  R  S  G  P  A  V  D  A  H  E  A  L  L  A  A  A  A  L  L  G  P  H  D  S  G  R  A  L  H  A
                                                       KpnI      NcoI      SalI
3001 TGCTCGGGGCCGCCGATGCCGCCTGGGTGCGGGGGGACGCGGGCGGGTACCTGGAGGCCATGGGGCGCGTCGACCCCACCCCGTACGGCGACGCCTTCGC
 473 L  L  G  A  A  D  A  A  W  V  R  G  D  A  G  G  Y  L  E  A  M  G  R  V  D  P  T  P  Y  G  D  A  F  A

3101 CTCCTACCGGGCGGGCATGTGCGCCGTGCTCGCCGGGCGGACCGCCGAGGGCCACGCCCTGCTCCGGCGGTGCCTCGACCCGGACGGGAGCGCCGAGGGG
 507   S  Y  R  A  G  M  C  A  V  L  A  G  R  T  A  E  G  H  A  L  L  R  R  C  L  D  P  D  G  S  A  E  G

3201 CACCCCGTCCTCCGCCGGTGCCCCGACCCCGCCGGTCTCCTCCGGGCCGGAGTCGCCGCGCTCGTGCTCGGGGAGGTGGCCGCCGCCTGCCGGATCGGGG
 540  H  P  V  L  R  R  C  P  D  P  A  G  L  L  R  A  G  V  A  A  L  V  L  G  E  V  A  A  A  C  R  I  G

3301 CGCGGGCGCTCGGGGCCGTACGGACCAGGGGGCCCGAGGTGTTGCTGCCGCAGGCCCTGGAGTACCTCGCCTACGCCGAGCTGCGGGCCGGGCTGCACGC
 573 A  R  A  L  G  A  V  R  T  R  G  P  E  V  L  L  P  Q  A  L  E  Y  L  A  Y  A  E  L  R  A  G  L  H  A

3401 GGCGGCCCGGGCCCACGCCCTGGAGGGGCTGCACGCCGCACGGCGGACCGGGCAGCCCAACAGCTCCGCCCGTCTGCACGCCGTCCTGGCCCTCGCCGCC
 607   A  A  R  A  H  A  L  E  G  L  H  A  A  R  R  T  G  Q  P  N  S  S  A  R  L  H  A  V  L  A  L  A  A

3501 TCCGTGGAAGGGCCCGGCGAGGCGTGCGCCGCCCACTGCGATGCCGCGCTGGCCGGTGCCGTACCGCACGGGCTCGCCCAGCCCGCGACCCTCGCCACCT
 640  S  V  E  G  P  G  E  A  C  A  A  H  C  D  A  A  L  A  G  A  V  P  H  G  L  A  Q  P  A  T  L  A  T

3601 GGGCCCGGGGCCGCGCCGACCTGGCCGCCGGGCGCCCCGAGGAGGCCGCCGCCCGGCTGGGGCCGCTCGTGCGCCCCGGACCCGGGCAGGGGCACTTCGC
 673 W  A  R  G  R  A  D  L  A  A  G  R  P  E  E  A  A  A  R  L  G  P  L  V  R  P  G  P  G  Q  G  H  F  A

3701 CGTCCGGACGCTCGCCGTGCCCTGTTACGTCGAGGCCGTCGTCCTCGGCGGGCGGGGCGGTGAACTCTCCTCCGTCGTTGACGAGTTCGCTGCCTGGGCC
 707   V  R  T  L  A  V  P  C  Y  V  E  A  V  V  L  G  G  R  G  G  E  L  S  S  V  V  D  E  F  A  A  W  A

3801 GACCGGACCGCCGATCCCCAGGCCCCCGCCCAGCTCGCCCGCTGCCGCGCCCTGACGGCCCCCGCCGACGAGGCCGACGCCCGGTATGCGGAAGCCCTCG
 740  D  R  T  A  D  P  Q  A  P  A  Q  L  A  R  C  R  A  L  T  A  P  A  D  E  A  D  A  R  Y  A  E  A  L

3901 CCCACCACGACCGGGCGGGCGGCGACTTCGAGCGGGCCCGCACCCTGCTCCTTTACGGGCAGTGGCTGCGCCGCCGTCGCCGTACCCGTGAGGCCCGCAC
 773 A  H  H  D  R  A  G  G  D  F  E  R  A  R  T  L  L  L  Y  G  Q  W  L  R  R  R  R  R  T  R  E  A  R  T

4001 CCCGCTGCGCGACGCCCTCGTCGCCTTCCAGCGCTGTTCGGCCCGCGCCTGGGCCGAGCGGGCGGGCGGGGAGTTACGGGCGGCGGGGGAGCCGGTCGCC
 807   P  L  R  D  A  L  V  A  F  Q  R  C  S  A  R  A  W  A  E  R  A  G  G  E  L  R  A  A  G  E  P  V  A

4101 GGGGCGGTACGGGACACGGGCGGCGACGGGGTGCTCTCCGCGCTCACCCCGCAACAGCAGCGCATCGCCCGCTGCGTCGCCGAAGGGGCCACCAACCGCG
 840  G  A  V  R  D  T  G  G  D  G  V  L  S  A  L  T  P  Q  Q  Q  R  I  A  R  C  V  A  E  G  A  T [N  R
                                           SalI
4201 AGGTCGCGCTCCGGCTCTCGCTCAGCCCGCGCACGGTCGACCACCACCTCCGCAACGTCTTCGCCGCCCTCTCCGTCCGCTCCCGCACCGAACTGGCCCG
 873 E  V  A  L  R  L  S  L  S  P  R  T  V  D  H  H  L  R] N  V  F  A  A  L  S  V  R  S  R  T  E  L  A  R

4301 GCTGCTCGGCCCCCAGACGTCCCCGATGCCTCGGACGTCCCGGAGTCCCCAGAGTCCCCAGAGTCCCCAGAGTCCGCAGGGGCCGTGACGGGCAGGTGGA
 907   L  L  G  P  Q  T  S  P  M  P  R  T  S  R  S  P  Q  S  P  Q  S  P  Q  S  P  Q  G  P End
```

FIG. 5—*Continued.*

and higher than that of any of the sequences analyzed by Wright and Bibb (48). This is due to an unusually high G+C content in the first and second positions of codons (84.5 and 63.6 mol%, respectively), whereas the third position of codons has the high G+C content expected for a *Streptomyces* gene (92.4 mol%). This very high G+C content is due to the high proportion of amino acids whose codons have either G or C in the first and second positions: alanine (23.3 mol%), arginine (10.4 mol%, of which only 2 of 98 codons are AGG), and glycine (10.3 mol%). The LipR sequence also contains a large number of leucine residues (13%), of which one is encoded by the rare TTA codon. Analysis of the LipR sequence with the REGULAT program (PC/Gene release 6.85) revealed the presence of a helix-turn-helix motif close to the C terminus of the protein; this program compares sequences with separate weight matrices obtained from helix-turn-helix motifs of positive and negative regulatory proteins, and LipR scored as a likely positive regulator. In addition, when the weight matrix proposed by Dodd and Egan (7) was used to analyze the LipR sequence it gave a greater than 90% probability of LipR being a DNA-binding protein (corresponding to a score of 4.2 standard deviations). A BLAST search (1) was conducted on the

```
                                                            KpnI.
4401  CGGGTGGTTGCGAGACCGTGATGACAAGTTCCGGGCAGACCCCTAGGTACCGACCGGGCGGTATGCCATTCTGCGGGCGTCAGGATCGCCCGGCCGATCT

4501  CCGGTGGAGGCCGCCATGCCCCAGCTCGTCCGTCCGCGGTTCGCGGTGCTGCACGGCACGCACCCCGCGGCGCCGTTGCCCCAGCGCTTCCGTTCCCTGG
   1     orf481 ——→fM  P  Q  L  V  R  P  R  F  A  V  L  H  G  T  H  P  A  A  P  L  P  Q  R  F  R  S  L

4601  CCGACCGCGAGGCCGTCGAGGTGCTGCACCGGGCCGCCCGGGTCCTCGTGGCCTCGCTCCCGGCCCTCACCGACCGGCTCGTGGAGGCCCTGTACGAGCA
  29  A  D  R  E  A  V  E  V  L  H  R  A  A  R  V  L  V  A  S  L  P  A  L  T  D  R  L  V  E  A  L  Y  E  Q

4701  GGAGCCCGGCTACCGGGCGGCCATCGACGCGGACCGGTCCGAGGTCTGGCAGGAGGTCCACCACTCCCTGCGCCACAACGTCGGCTCGCTGCTCCAGCCC
  63   E  P  G  Y  R  A  A  I  D  A  D  R  S  E  V  W  Q  E  V  H  H  S  L  R  H  N  V  G  S  L  L  Q  P

4801  AAGGAGTTCCGCGACGCCGCCCACCGCACGTCCCGCTGGATCGGTGAGATCCGCGCCGGACAGGGCGTCCCGCTCGACGCCGTCCTGCACGCCTTCCGGA
  96   K  E  F  R  D  A  A  H  R  T  S  R  W  I  G  E  I  R  A  G  Q  G  V  P  L  D  A  V  L  H  A  F  R
                            SalI
4901  TGGGCGGGGCGATGGTCTGGCAGGACCTGGTCGACGAGACGGCCCGCCGCGGCCCCGACGACGTACGGCTGCTCGTCCACGTCGCCGCCGACGTGTGGAA
 129  M  G  G  A  M  V  W  Q  D  L  V  D  E  T  A  R  R  G  P  D  D  V  R  L  L  V  H  V  A  A  D  V  W  N
        SalI .
5001  CTTCGTCGACGAGCACTGCGGCATCGTCGCGGACGCCTACCGGCAGGCCGAGCGCCGGCTGACCTGGCAGCGCGAGAACCGGCAGCGGCTGGTGATCGCG
 163   F  V  D  E  H  C  G  I  V  A  D  A  Y  R  Q  A  E  R  R  L  T  W  Q  R  E  N  R  Q  R  L  V  I  A

5101  GCGCTGCTCGACGGGACCGCCCGGATCGCGGACCTCAGCGACGCGGCGGCCATGCTGGGCCTGCCGGAACAGGGGCGGTACGCGGTGCTCGCGGTGGCCT
 196   A  L  L  D  G  T  A  R  I  A  D  L  S  D  A  A  A  M  L  G  L  P  E  Q  G  R  Y  A  V  L  A  V  A

5201  CCGTACGGCGCGGCGACCCCGGCCCCGTACGGCAGTCCCAGCCGGAACCCGTCCGGCCGCTGCTGAACCTGCCGTCCGCCCTGCCGCCGCCGGGCGAGCC
 229  S  V  R  R  G  D  P  G  P  V  R  Q  S  Q  P  E  P  V  R  P  L  L  N  L  P  S  A  L  P  P  P  G  E  P

5301  CCGGTACGGCCGGGAGGAGGCGGGGGTGGCGACCCCGGCCCCGCTCCCGGGCACCGCTCCGGAGGAGCCGCCCGGACCGGACGTCCGGGTGGCCGAGGCC
 263   R  Y  G  R  E  E  A  G  V  A  T  P  A  P  L  P  G  T  A  P  E  E  P  P  G  P  D  V  R  V  A  E  A

5401  CCGGCCCCGTACGCCGGACCTGCCGGGCCACCGGGCACCGCCGCCCCCCTCTGGCACACCGGCCTCGACGCCGAGTTCGCCATCCTCCGGCTCACCGGCG
 296   P  A  P  Y  A  G  P  A  G  P  P  G  T  A  A  P  L  W  H  T  G  L  D  A  E  F  A  I  L  R  L  T  G
                      SphI                                                    SalI .
5501  AACCGGGCGACCCCGGCGAGCTGCATGCCATCGCCGCCGCCCTGGACGCCCCCGACGGACCCGGGCCGGGATCGGCTCCGCCGTCGACGGGCTGGCCGC
 329  E  P  G  D  P  G  E  L  H  A  I  A  A  A  L  D  A  P  D  G  T  R  A  G  I  G  S  A  V  D  G  L  A  A

5601  GCTCGGGGACGCCCGGCGGCTGGCGGAGACCGCGCTGCGGGCCTGCCCGGCCTCCGGGGGCACGGTCGTGCTCGACGAGCACCTCCCGGACGCCCTCGTC
 363   L  G  D  A  R  R  L  A  E  T  A  L  R  A  C  P  A  S  G  G  T  V  V  L  D  E  H  L  P  D  A  L  V

5701  GTCTCCTCCCCGGCCCTCGGATCGGAGGCCGAGGTGGTGATCGAGGACAACGGCGGCGGCATGGACCCCGAACGGCTGCGCCGCATCCTGCGCGGCGAGG
 396   V  S  S  P  A  L  G  S  E  A  E  V  V  I  E  D  N  G  G  G  M  D  P  E  R  L  R  R  I  L  R  G  E

5801  CCACCACCCCCTCCACCGGCATCGGCCTGCTCAACGTGGACGAGCGGCTGCGCCAGGTGTACGGGGACGGGTACGGGCTCGTCATCGAGACCGGGGTCGG
 429  A  T  T  P  S  T  G  I  G  L  L  N  V  D  E  R  L  R  Q  V  Y  G  D  G  Y  G  L  V  I  E  T  G  V  G

5901  CGCGGGCATGAAGATCACGCTCCGGCTGCCCAAGTACCGCGCGGGCGTGCACGGCTCATGAGCCCCGGGCCCCGTCCGGGCCCGGCTTTCTCAGCAGAGA
 463   A  G  M  K  I  T  L  R  L  P  K  Y  R  A  G  V  H  G  S End

6001  TGGACGGCCAGGTGCCCCAGTGGCAGCCCCAGTTGCCACGCCGGGGTCCACACCTGGACCTCCCCCTCGGTGTCGTGCCCGCCCCCGGCCACCGGCTCCC

6101  GGGGCGTGGTCACGATC
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FIG. 5—*Continued.*

LipR sequence; the only high-scoring sequence obtained in this search was the product of a gene that has been only partially sequenced and that is located just downstream of the *S. albus* G *lipA* homolog (GenBank accession no. U03114). In addition, the BLAST search revealed a large number of protein sequences which gave a lower, but significant, local alignment score; in all cases it was the same region of LipR which was responsible for the local alignment, and this was precisely

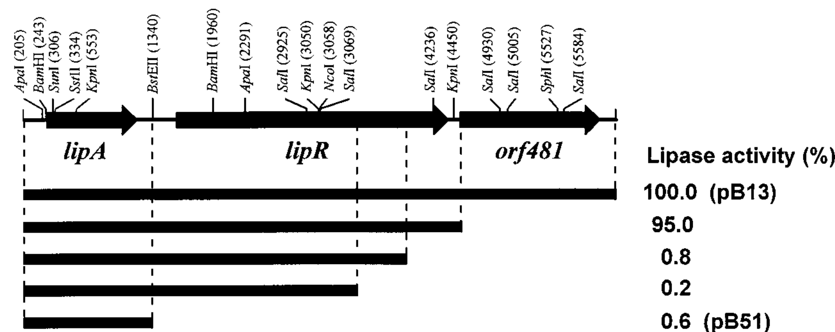

FIG. 6. Map of the pB13 insert and phenotype of exonuclease III-generated deletions. The upper part of the figure shows the restriction map of the sequenced pB13 insert and the included genes. Numbers in parentheses refer to the position of the restriction sites in the insert; not all *Apa*I, *Sun*I, or *Sst*II sites are shown. Filled bars in the lower part of the figure correspond to the amount of insert DNA remaining after treatment with exonuclease III. The activity found in the supernatant after 96 h of growth is shown as a percentage relative to that of a 1326(pB13) control culture; the activity obtained from supernatants of 1326(pB51) cultures is also shown.

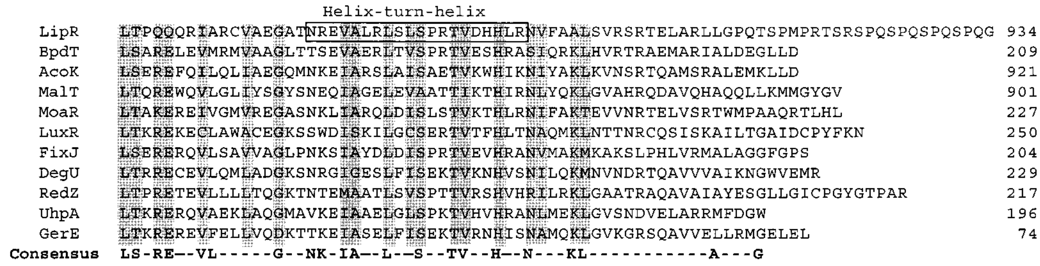

FIG. 7. Alignment of the C-terminal putative DNA-binding domain of LipR with the homologous domains of other known transcriptional activators. Positions showing strong conservation are shaded. The consensus shown below the aligned sequences is that of the class 3 family of response regulators (33a) and associated regulatory proteins, which is equivalent to the UhpA/LuxR superfamily of regulators as defined in the PROSITE database (2, 11a). The GenBank or Swiss-Prot accession numbers for the different sequences are U85412 (*Rhodococcus* sp. BpdT), U10553 (*K. pneumoniae* AcoK), AE000418 (*E. coli* MalT), D49928 (*Klebsiella aerogenes* MoaR), Y00509 (*Vibrio fischeri* LuxR), P10958 (*Rhizobium meliloti* FixJ), P13800 (*Bacillus subtilis* DegU), Y07902 (*S. coelicolor* RedZ), M89479 (*E. coli* UhpA), and P11470 (*B. subtilis* GerE). The helix-turn-helix motif identified in LipR by the REGULAT program is boxed.

the C-terminal region which includes the helix-turn-helix motif. Most of these proteins are known response regulators, whereas others are transcriptional activators which possess evolutionarily related C-terminal DNA-binding domains and lack the receiver module of response regulators (33a). These proteins have been grouped together, based on the similarity of their DNA-binding domains, in the UhpA/LuxR superfamily of bacterial regulators (2, 11a) or as regulators having a class 3 DNA-binding domain (33a); an alignment of the putative DNA-binding domain of LipR and the DNA-binding domains of some of these known regulators is shown in Fig. 7. Most regulators belonging to this superfamily have a relatively small size (around 200 to 250 amino acids) with the exception of MalT, the activator of the maltose utilization regulon of *E. coli* (5a), and AcoK, the recently described activator of acetoin catabolism of *Klebsiella pneumoniae* (33b), both of which have sizes similar to LipR (901 and 921 amino acids, respectively). LipR shows significant similarity to both MalT (19% identity, 10% similarity) and AcoK (19% identity, 11% similarity), although it is not as high as that exhibited between MalT and AcoK themselves (25% identity, 15% similarity; data not shown); in particular, LipR lacks the nucleotide-binding motif present in the N-terminal parts of these two activators, which are ATP dependent (33b, 35a). All these findings provide support for the notion that LipR is a DNA-binding protein whose function is to activate transcription at the *lipA* promoter.

***bldA* dependence of *lipA* expression is mediated by LipR.** As mentioned in the previous section, codon usage analysis of *lipR* revealed the presence of a leucine residue encoded by the unusual TTA codon (amino acid position 831 of the LipR sequence [Fig. 5]). Since the *bldA*-encoded tRNA is the only one capable of efficiently translating TTA codons (26), lipase expression should be *bldA* dependent if LipR is indeed required for *lipA* transcription. To test this, pB13 was introduced into the *S. coelicolor bldA39* mutant, J1700, and into the isogenic *bldA*$^+$ strain, J1501, and lipase production was assayed qualitatively on olive oil-rhodamine B plates. J1501(pB13) gave rise to large halos of lipolysis surrounding the colonies, whereas no halos were seen around the J1700(pB13) colonies. No halos were seen when either J1501 or J1700 carried the vector pIJ486 (data not shown). Therefore, as expected, lipase expression is *bldA* dependent.

Timing of lipase synthesis is not determined by the *bldA* dependence of LipR. In order to test whether the timing of lipase gene expression was due solely to its dependence on the *bldA* gene product, a plasmid carrying *lipA* and *lipR* (but not *orf481*) was constructed in which the TTA codon inside *lipR* had been replaced by another leucine codon (CTC) by means of site-directed mutagenesis (plasmid pB94 [Table 1]); an identical plasmid that carried the wild-type (TTA-containing) *lipR* sequence was constructed as a control (pB96 [Table 1]). Both plasmids were introduced into *S. coelicolor* J1501 and J1700, and lipase production was assayed qualitatively on olive oil-rhodamine B plates; as expected, J1501 carrying either plasmid showed halos of lipolysis, whereas only J1700(pB94) was lipolytic. The temporal pattern of *lipA* transcription by these four strains was analyzed; as seen in Fig. 8, abundant *lipA* transcripts were found in RNA preparations from stationary-phase cultures of both J1501(pB94) and J1501(pB96), and this correlated with lipase activity appearing in the medium only during stationary phase (data not shown). *lipA* transcription by J1700(pB94) cultures and lipase activity in the supernatant were also mainly restricted to the stationary phase (Fig. 8); on

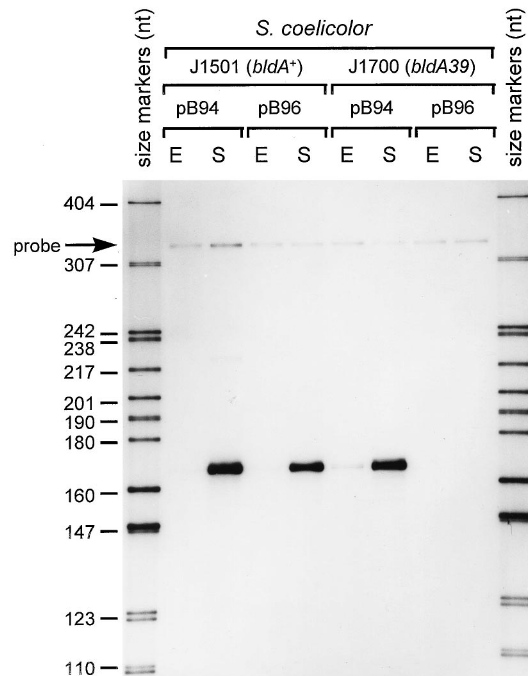

FIG. 8. Transcription of *lipA* in *S. coelicolor* J1501 (*bldA*$^+$) and J1700 (*bldA39*) carrying different plasmids. Plasmids pB94 and pB96 are identical constructs which carry only the *lipA* and *lipR* genes; in pB94 the single TTA codon inside *lipR* has been replaced by the alternate CTC codon, whereas pB96 retains the TTA codon. Symbols are the same as in Fig. 3.

prolonged exposure of the autoradiographs a small number of *lipA* transcripts could be detected in exponential-phase RNA preparations from J1501 cultures carrying either plasmid and from J1700(pB94) cultures (data not shown). On the other hand, J1700(pB96) cultures were unable to secrete lipase and no *lipA* transcripts could be detected in either exponential- or stationary-phase RNA preparations from these cultures, even after prolonged exposure of the autoradiographs. This experiment confirmed that *lipA* transcription is dependent on the *lipR* product.

Replacement of the TTA codon by the alternate CTC codon in *lipR* alleviated *bldA* dependence but not the apparent growth phase dependence of *lipA* transcription (Fig. 8); this result could be explained if transcription of *lipR* were itself growth phase dependent and mostly restricted to the stationary phase. In order to find out whether this was the reason for delayed expression of *lipA* in cultures carrying pB94, the version of *lipR* lacking the TTA codon was placed under the control of the *tipA* promoter region, after the introduction of a suitable *Nde*I site at the start codon in order to allow cloning into the expression vector pIJ6021, which resulted in plasmid pB99. This plasmid was introduced into *S. lividans* 1326 carrying pB100, which consists of the pJV1-derived vector pB48 (conferring hygromycin resistance [39]) into which the *lipA* gene and promoter had been cloned in the absence of any *lipR* sequences (Table 1). As a control, pIJ6021 was also introduced into *S. lividans* 1326(pB100). *S. lividans* 1326 carrying both pairs of compatible plasmids was assayed for lipase production on olive oil-rhodamine B plates; colonies of *S. lividans* carrying pIJ6021 plus pB100 were unable to form halos of lipolysis, whereas those carrying pB99 plus pB100 showed large fluorescent halos, indicating that the *lipR* product was capable of activating *lipA* transcription in *trans*. Liquid cultures of *S. lividans* 1326(pB99/pB100) revealed that lipase was secreted from the start of the exponential growth phase and that addition of thiostrepton significantly increased the amount of lipase in the medium, most of which accumulated during the exponential growth phase (Fig. 9); as expected, control cultures of 1326(pIJ6021/pB100) were unable to secrete lipase, irrespective of *tipA* induction.

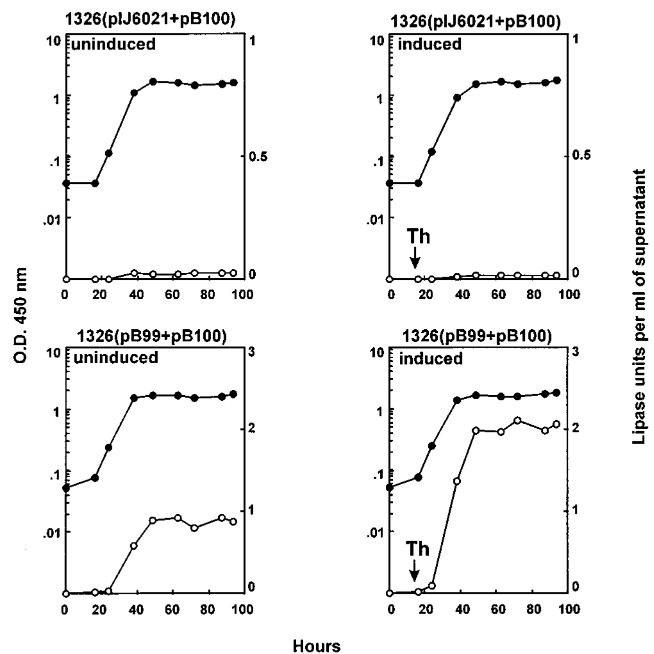

FIG. 9. Growth of *S. lividans* 1326 carrying different plasmids (●) and lipase activity in the supernatant (○). pIJ6021 is a pIJ101-derived expression vector (44); pB100 is a pJV1-derived plasmid into which the *lipA* gene and promoter have been cloned; pB99 consists of the *lipR* gene cloned into pIJ6021 under the control of the inducible *tipA* promoter. Induction of the *tipA* promoter was achieved by adding 5 μg of thiostrepton (Th) ml^{-1} at the time indicated by an arrow. O.D., optical density.

DISCUSSION

Even though a significant number of prokaryotic lipase genes have been cloned (18), there have been few studies aimed at elucidating the molecular mechanisms which regulate their expression; there is, however, evidence that some lipase genes are subject to transcriptional regulation. Thus, in *Staphylococcus aureus* S6C a chromosomal mutation in the *xpr* locus, unlinked to the lipase structural gene, eliminated extracellular lipase production (40); the effect of this mutation was shown to be at the transcriptional level, although it was not specific, since transcription of several genes encoding exoproteins was affected (13). In addition, gene fusion experiments have shown that lipase synthesis in *Acinetobacter calcoaceticus* BD413 is regulated at the transcriptional level, and the existence of a regulator for lipase gene transcription has been inferred, although it has not yet been identified (21). The results presented in this paper show that transcription from the *S. exfoliatus* M11 *lipA* promoter requires the presence of a contiguous downstream gene, *lipR*, since *lipA* transcripts could be detected only in its presence; this requirement appeared to be specific for transcription from the *lipA* promoter, since it was overcome when *lipA* was fused to the promoter regions of other streptomycete genes. The presence of a TTA codon in *lipR* allowed us to show that *lipA* transcription was dependent on the LipR protein, since no transcripts could be observed in a *S. coelicolor* BldA$^-$ mutant when the wild-type *bldA*-dependent *lipR* gene was present, whereas normal transcription was observed in the presence of a *bldA*-independent version of *lipR*; in addition, *lipR* provided in *trans* directed high levels of lipase synthesis which responded to the timing of controlled *lipR* induction (Fig. 9). The most relevant characteristic of the LipR sequence is the presence of a putative DNA-binding domain at its C terminus; sequence comparison reveals that this domain shows significant similarity to the highly conserved C-terminal DNA-binding domains of a large family of proteins which comprises mostly, but not exclusively, response regulators (2, 33a). Other members of this family include transcriptional activators with a homologous DNA-binding domain that lack the receiver module of response regulators, such as members of the LuxR subfamily of cell density-responsive regulators (11a) as well as other transcriptional activators, such as MalT; in the case of MalT it has been shown that this C-terminal domain is sufficient for DNA binding (44a). MalT was the only activator belonging to this family with a disparate, larger size (33a) until the recent description of AcoK, a MalT homolog of similar size (33b) which also functions as a transcriptional activator. LipR shows significant similarity to these two activators which is not restricted to the DNA-binding domain. Therefore, LipR appears to be the third member of a subfamily of proteins of similar size which belong, based on their DNA-binding domains, to the UhpA/LuxR superfamily of regulators (2, 33a). All these lines of evidence support the notion that *lipR* encodes a transcriptional activator of the *lipA* promoter. The presence of a gene encoding a LipR homolog downstream of the *lipA* gene of *S. albus* G suggests that the close physical linkage of lipase genes and their cognate regulatory genes in *Streptomyces* might be common; in fact, Southern hybridization experiments

have revealed sequences homologous to *lipR* in the same *Streptomyces* strains previously shown to hybridize to a *lipA*-specific probe (6) but not in strains lacking *lipA*-homologous sequences (unpublished results). Given the close physical association of the *lipA* and *lipR* genes, LipR is likely to be a specific regulator of lipase synthesis rather than a general one, as in the case of the *S. aureus* regulator encoded in the unlinked *xpr* locus (13). Therefore, the identification and sequencing of LipR appears to be the first report of a specific transcriptional regulator of lipase gene expression in prokaryotes.

The results presented here show that transcription of *lipA* is growth phase dependent under the conditions used and that this growth phase dependence is mediated by *lipR*. The apparent growth phase dependence was evidenced by the increase in lipase activity and protein during stationary phase, which correlated with the observation that *lipA* transcripts were abundant only in RNA preparations obtained during the stationary phase; it should be noted, however, that there are other possibilities which could account for these observations. An increase in the plasmid copy number upon entry into stationary phase, for example, could lead to similar results. This possibility is unlikely, however, since it has been shown that the copy number of pIJ101-based vectors in batch culture conditions is not significantly altered after entry into the stationary phase (in fact copy number is higher during the exponential growth phase [48a]); these changes could hardly account for the difference in the number of *lipA* transcripts between exponential- and stationary-phase cultures; in addition, growth phase-dependent lipase synthesis was also observed with an SCP2*-derived vector, whose copy number is tightly controlled and maintained at one to two copies per chromosome, even in stationary-phase cultures (3a). Another possible explanation for the results obtained in this work could be that a change in the stability of *lipA* transcripts, rather than growth phase-dependent initiation of transcription at the *lipA* promoter, is responsible for their increased abundance during stationary phase; if this were so, then the stabilization of *lipA* transcripts should be mediated by LipR. This is an unlikely possibility, however, since LipR is a homolog of known transcriptional activators with a well-conserved DNA-binding domain.

Since expression of the *S. exfoliatus* M11 lipase is *bldA* dependent, a parallel can be drawn to the expression of genes encoding biosynthetic enzymes of the actinorhodine and undecylprodigiosin pathways of *S. coelicolor* A3(2) (3, 12, 43); in these two well-studied cases, specific transcriptional activators (the products of the *act*II-ORF4 and *redD* genes) turn on their respective pathway-specific genes during the transition into stationary phase, since their genes are in turn transcribed in a growth phase-dependent fashion (12, 43) in response to an as yet unidentified signal (3). In the case of actinorhodine, *bldA* dependence is caused by the presence of a TTA codon in *act*II-ORF4 (9), whereas in the case of undecylprodigiosin, *bldA* dependence is mediated by RedZ, an additional activator of *redD* transcription. The *redZ* gene has a TTA codon (47). Interestingly, the RedZ sequence (GenBank accession no. Y07902) reveals a putative DNA-binding domain similar to that of LipR (Fig. 7), and the position of the TTA-encoded leucine in both sequences is close to the C terminus (in the case of RedZ it is inside the DNA-binding domain, and in the case of LipR it is a few amino acids before it). The *bldA* dependence of LipR does not appear to be the main cause for growth phase-dependent lipase expression, since replacement of the single TTA codon in *lipR* by an alternate leucine codon resulted in the same temporal pattern of *lipA* transcription; this is in agreement with the observation that the timing of *act*II-ORF4 and *redZ* transcription, rather than their *bldA* dependence, is the main cause for growth phase-dependent actinorhodine and undecylprodigiosin synthesis (12, 47). Clearly, it will be necessary to study *lipR* transcription in more detail in order to confirm whether its transcription is growth phase dependent and to explore the possibility that it shares other regulatory aspects with *act*II-ORF4 and *redD*, such as in vitro transcription by RNA polymerase holoenzyme carrying σ^{hrdD} (11) or conditional dependence on the product of the pleiotropic gene *afsR* (10).

Why should lipase expression be limited to the stationary phase? One possible explanation might be related to the recent finding that *Streptomyces* spp. are perhaps unique among prokaryotes in using triacylglycerols as storage compounds; it has been speculated that these serve as possible carbon sources for antibiotic synthesis (32). These triacylglycerols are accumulated intracellularly in membrane-bound structures during the exponential growth phase (33), and it is clear that the first step in their utilization should involve lipolytic enzymes. The fact that the *Streptomyces* lipases are extracellular does not necessarily mean that triacylglycerols accumulated intracellularly might not be available to them, since under normal conditions for *Streptomyces* growth (surface-grown cultures) lysis and cannibalism of the substrate mycelium play roles in providing the differentiating and antibiotic-producing parts of the colonies with nutrients (5).

In conclusion, we have shown that transcription of the cloned *S. exfoliatus* M11 *lipA* gene is *bldA* dependent and that this dependence is mediated by a putative transcriptional activator of the *lipA* promoter, encoded by the *lipR* gene downstream of *lipA*, which has a single TTA codon. The apparent growth phase-dependent expression of *lipA* is not a consequence of *bldA* dependence and is also mediated by LipR.

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