Lipase Expression in *Pseudomonas alcaligenes* Is Under the Control of a Two-Component Regulatory System

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Received 17 July 2007/Accepted 27 December 2007

Preliminary observations in a large-scale fermentation process suggested that the lipase expression of *Pseudomonas alcaligenes* can be switched on by the addition of certain medium components, such as soybean oil. In an attempt to elucidate the mechanism of induction of lipase expression, we have set up a search method for genes controlling lipase expression by use of a cosmid library containing fragments of *P. alcaligenes* genomic DNA. A screen for lipase hyperproduction resulted in the selection of multiple transformants, of which the best-producing strains comprised cosmids that shared an overlapping genomic fragment. Within this fragment, two previously unidentified genes were found and named *lipQ* and *lipR*. Their encoded proteins belong to the NtrBC family of regulators that regulate gene expression via binding to a specific upstream activator sequence (UAS). Such an NtrC-like UAS was identified in a previous study in the *P. alcaligenes* lipase promoter, strongly suggesting that LipR acts as a positive regulator of lipase expression. The regulating role could be confirmed by down-regulated lipase expression in a strain with an inactivated *lipR* gene and a threefold increase in lipase yield in a large-scale fermentation when expressing the *lipQR* operon from the multicopy plasmid pLAFR3. Finally, cell extracts of a LipR-overexpressing strain caused a retardation of the lipase promoter fragment in a band shift assay. Our results indicate that lipase expression in *Pseudomonas alcaligenes* is under the control of the LipQR two-component system.

Lipases (triacylglycerol acylhydrolase; EC 3.1.1.3) are produced by a wide variety of living organisms. Most microbial lipases are secreted into the extracellular medium, which makes them easily recoverable for use in industrial applications. Of particular interest are lipases made by *Pseudomonas* species that have properties compatible with use in household detergents (52), synthesis of pharmaceuticals or agrochemicals (29, 57), and processing of fats (24, 60).

The production of lipases by fermentation, however, is complicated and poorly understood. Some processes have been developed empirically, but the control of lipase synthesis and the reproducibility of the processes are unsatisfactory. Furthermore, the induction of lipase expression by various inducer molecules is not understood. Lipase expression in fungi, like *Penicillium simplicissimum* (52), *Geotrichum candidum* (47), *Aspergillus japonicus* (56), *Beauveria bassiana* GK 2116 (19), and *Oospora fragrans* (46), can be induced with oils and fatty acids. In other species, such as *Sulfolobus shibatae* (20) and *Malassezia furfur* (41), lipase expression can be stimulated by various types of detergents, such as Tween (poly-oxethylene-sorbitan). For some *Pseudomonas* species (55), fatty acid alcohols are mentioned as the inductive component. For *Acinetobacter calcoaceticus* BD413, aliphatic alkane *n*-hexadecane (23) has been identified as an inducer. In recent years, lipase expression of *Pseudomonas alcaligenes* M-1 has been studied extensively (13), and a fed-batch fermentation process was developed. It was found that lipase synthesis can be switched on by a limited number of natural oils, such as soybean oil, and some unsaturated fatty acids, such as oleic acid. A significant synthesis of lipase was observed only for the combination of a batch phase based on a minimal citrate medium and a feed phase based on soybean oil. Expression of lipase was observed only after the start of the feed with soybean oil (13).

Numerous bacterial lipase genes have been identified and sequenced in the past (15, 21). Nevertheless, the lipase expression transcriptional regulation mechanism in particular is poorly understood. Almost no reports about the transcriptional regulation of lipase genes from pseudomonads are available. It is known that lipases from a number of *Pseudomonas* and *Burkholderia* species are expressed from a unique type of operon, where the structural gene for lipase (*lipA*) is followed by a gene coding for a helper protein (*lipB*). In the past, this lipase helper protein—LipB—has been investigated for its suspected role in the regulation of lipase expression (12). It is now generally accepted that the lipase helper protein plays a role in periplasmic lipase folding and not in transcriptional regulation (22). For *Acinetobacter calcoaceticus* BD413 (23) and *Rhizobium etli* (53), the presence of a σ70 (RpoA-dependent) promoter as the lipase transcriptional regulon has been reported. For *P. alcaligenes* M-1, the transcription start of the lipase operon was mapped (7) downstream of a typical σ54-type promoter with the characteristic GG-N10-GC sequence at transcription start positions −12 to −24 (9, 30). Also, for *Pseudomonas aeruginosa* (22) and *Pseudomonas* species M-12-33 (36), a σ54 (RpoN-dependent) promoter in front of the lipase operon has been postulated based on sequence data. These promoters operate in conjunction with an upstream activator sequence (UAS) essential for the binding of a regulatory protein. Upon the binding of the regulatory protein to...
this UAS and to σ54 RNA polymerase, the expression of the gene is induced. Within the upstream region of the lipase promoter region of \( P. \) \textit{alcaligenes}, a sequence that is homologous to the Nif type of UASs (consensus, TGT-N10-ACA) was identified (7). Mutational analysis of this UAS confirmed an involvement in lipase regulation (7).

One rational approach for finding regulatory proteins is to search for negative phenotypes followed by complementation using libraries of \( P. \) \textit{alcaligenes} genomic DNA. However, mutagenesis of \( P. \) \textit{alcaligenes} M-1 with physical and chemical agents yielded lipase-negative phenotypes at high frequency, resulting in too many mutants to work with (data not shown). Consequently, in order to identify factors essential in the regulation of lipase from \( P. \) \textit{alcaligenes}, we have developed a selection for positive phenotypes—the phenotype enhancement method. A cosmid library of random chromosomal fragments from \( P. \) \textit{alcaligenes} was introduced into a high-producing lipase strain and subsequently a sensitive screening for hyperproducing strains was done under various growth conditions. One of the elements found, a two-component regulatory system, is a subject of this study.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, culture conditions, and chemicals.** For this study, bacterial strains from the species \( \text{Pseudomonas alcaligenes} \) and \( \text{Escherichia coli} \) were used. The introduced genetic modifications and plasmids used are shown in Table 1.

All bacterial strains listed in Table 1 were propagated in 2× \( T \) \( Y \) (16 g/liter Bacto tryptone, 10 g/liter Bacto yeast extract, 5 g/liter \( \text{NaCl} \), pH 7.0) as a liquid or solid medium, unless otherwise stated. Two screening tests containing minimal salt medium (for composition, see reference 14) were supplemented with either 0.2% soybean oil (test I) or 0.5% sodium lactate (test II). Screening test III consisted of brain heart infusion (BHI) medium (37 g/liter BHI; Difco) supplemented with 17 mM \( \text{n-hexadecane} \).

Bacterial strains containing plasmids or cosmids were grown either in medium with physical and chemical agents yielded lipase-negative phenotypes at high frequency, resulting in too many mutants to work with (data not shown). Consequently, in order to identify factors essential in the regulation of lipase from \( P. \) \textit{alcaligenes}, we have developed a selection for positive phenotypes—the phenotype enhancement method. A cosmid library of random chromosomal fragments from \( P. \) \textit{alcaligenes} was introduced into a high-producing lipase strain and subsequently a sensitive screening for hyperproducing strains was done under various growth conditions. One of the elements found, a two-component regulatory system, is a subject of this study.

### TABLE 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>Restriction negative, modification positive ( \text{Ps93} )</td>
<td>14</td>
</tr>
<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>High-lipase-producing isolate of strain M-1 ( \text{Ps537} )</td>
<td>7</td>
</tr>
<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>Neo’ [pJRDlipAB] ( \text{Ps770} )</td>
<td>This study</td>
</tr>
<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>( \text{lipA} ) ( \text{Ps824} )</td>
<td>This study</td>
</tr>
<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>( \text{Ps1018} )</td>
<td>This study</td>
</tr>
<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>( \text{Ps770} ) with cosmid 201; Neo’ ( \text{Ps1029} )</td>
<td>This study</td>
</tr>
<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>( \text{Ps1030} ) with ( \text{pLAFR3} ); Neo’ ( \text{Ps1034} )</td>
<td>This study</td>
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<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>( \text{Ps1036} )</td>
<td>This study</td>
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<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>( \text{Ps537} ) with cosmid 505; Neo’ ( \text{Ps1039} )</td>
<td>This study</td>
</tr>
<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>( \text{Ps1040} )</td>
<td>This study</td>
</tr>
<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>( \text{Ps1041} )</td>
<td>This study</td>
</tr>
<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>( \text{Ps1042} )</td>
<td>This study</td>
</tr>
<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>( \text{Ps1049} )</td>
<td>This study</td>
</tr>
<tr>
<td>( P. ) \textit{alcaligenes} strains</td>
<td>( \text{Ps1100} )</td>
<td>This study</td>
</tr>
<tr>
<td>( E. ) \textit{coli} strains</td>
<td>( F' ) ( \text{mcrA} ) ( \Delta(\text{mrr-hsd-RMS}) ) ( \Delta(\text{lacX74 deoR recA1 araD139}) ) ( \Delta(\text{ara-leu}) ) ( \text{K802} )</td>
<td>17</td>
</tr>
<tr>
<td>( E. ) \textit{coli} strains</td>
<td>( \text{galU galK rpsL Str} ) ( \text{endA1 mupG} ) ( \text{DH10B} )</td>
<td>42</td>
</tr>
<tr>
<td>( E. ) \textit{coli} strains</td>
<td>( \text{psd22 galK2 galT22 mcrB1 metB1 supE44} ) ( \text{Top10} )</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

\( \text{pLAFR3} \) (strain \( \text{Ps1029} \)) is described in reference 49. Str’, streptomycin resistance.
loration with the QIAfilter plasmid midi kit, both according to Qiagen’s instructions. Plasmids and cosmids were transformed to E. coli as described by Calvin and Hanawalt (5). For P. alcaligenes transformation, the method of Wirth et al. (59) was applied, with the modification that all treatments were performed at room temperature. Purification of PCR products was done using the PCR purification kit from Qiagen according to the supplier’s instructions. For Southern hybridizations (48), detection of DNA-containing filters was executed with DNA probes labeled with the ECL labeling kit from Gibco-BRL (following the instructions of the supplier).

**RNA isolation, cDNA preparation, and qRT-PCR.** Total RNA was isolated with a High Pure RNA isolation kit (Roche) from the P. alcaligenes cells collected from minimal tributyrin plates. Total RNA was quantified with an ND-1000 spectrophotometer (NanoDrop). Reverse transcription was conducted in a total volume of 20 μl. Random nanomers (2.5 μl of 1.65 μg/μl; Isogen) were mixed with 2.5 μg isolated RNA and 2 μl 10 mM deoxyribonucleoside triphosphate mix, incubated for 5 min at 65°C, and cooled on ice. Subsequently, 4 μl 5×-concentrated first-strand buffer (Invitrogen), 1 μl 100 mM dithiothreitol, 1 μl RNaseOUT (40 U/μl; Invitrogen), and 1 μl SuperScriptIII reverse transcriptase (200 U/μl; Invitrogen) were added, and the total volume was adjusted with diethyl pyrocarbamide-treated water to 20 μl and kept at 25°C for 5 min. A reverse transcription reaction was performed at 55°C for 60 min and was stopped by heating at 70°C for 15 min. Synthesized cDNA was further used in quantitative real-time PCR (qRT-PCR) that was carried out in 96-well microtiter plates in a final reaction volume of 20 μl by use of a Cycler iCycler iQ real-time PCR instrument (Bio-Rad). Each reaction mixture consisted of 1 μl cDNA, 10 μl iQ SYBR green supermix (Bio-Rad), 1 μl forward primer (10 μM), 1 μl backward primer (10 μM), and 7 μl water. Reaction mixtures were initiated with a 4-minute incubation at 95°C followed by up to 40 cycles at 95°C for 10 s (denaturation) and 60°C for 45 s (primer annealing/extension). The increase in fluorescence was measured automatically during PCR. Oligonucleotide pair primers for the lipA gene and the 16S rRNA housekeeping gene were designed with PerlPrimer software. Cycle threshold values were determined using the software provided with the thermal cycler. All cDNA samples were amplified in triplicate and normalized against a triplicate of the housekeeping gene 16S rRNA in the same plate.

**Protein expression, preparation, and purification.** E. coli DH10B, and P. alcaligenes DH10B, and P. alcaligenes M-1 is not able to grow on glucose as a single carbon source (13), other carbon sources were used as described in Materials and Methods. The media for screening tests I, II, and III were used to grow cosmids containing random fragments of P. alcaligenes chromosomal DNA. Subsequently, 531 independent cosmids could be transferred to P. alcaligenes strain Ps93 (restriction negative, modification positive), giving a cosmid library in a lipase-overproducing strain, Ps770, which harbors a multi-copy plasmid (neomycin-resistant [Neo] pJRDLipAB [13]) carrying the lipA and lipB genes, followed by quantitative lipase assays. In E. coli K802, 754 transformants (Tot) were collected with cosmids containing random fragments of P. alcaligenes chromosomal DNA. Subsequently, 531 independent cosmids could be transferred to P. alcaligenes strain Ps93 (restriction negative, modification positive), giving a cosmid library in P. alcaligenes. Cosmid DNA was isolated from these 531 Ps93 strains, and all cosmids were transformed individually to strain Ps770 by electroporation. In total, 485 cosmids could be successfully introduced into the lipase-overproducing strain Ps770, resulting in colonies that are both Neo and Tet. A screen involving three different microtiter tests was developed, and all 485 cosmid-containing strains were tested in duplicate and judged on their lipase activity.

The initial screening led to the selection of 42 strains with a significantly high lipase expression in one of the three tests compared to the Ps1029 strain containing the empty pLAFlR3 vector (49). The examination of these 42 strains was repeated in quadruplicate. Out of them, 20 strains were found to score considerably higher than strain Ps1029 in the soybean oil test. The 20 cosmids strains were ranked by their lipase activity (clarification zone on tributyrin oil agar plate/optical density at 600 nm) obtained in 10×-diluted minimal salt medium supplemented with soybean oil. Table 2 presents the results for four cosmids (505, 71, 201, and 726). DNA from all 20 lipase-
staining cosmids was isolated and transformed via electroporation to *P. alcaligenes* strain Ps537, which carries only a single copy of the lipase operon in the chromosome. Four of the resulting strains, Ps1039, Ps1040, Ps1041, and Ps1042 (containing cosmids 505, 71, 726, and 201, respectively), gave a clarification zone (halo) much larger than that of the wild-type strain on a tributyrin oil plate (Fig. 1). This indicates that the four cosmids harbor either a factor that stimulates the lipase expression or an esterase or lipase gene that hydrolyzes tributyrin oil. This latter possibility was ruled out by testing the four cosmids in a lipase-negative strain, Ps824, which carries a deletion of the lipase gene. None of the four cosmids showed hydrolysis of tributyrin oil after transfer to strain Ps824 (data not shown).

**Characterization and analysis of four lipase-stimulating cosmids.** The four lipase-stimulating cosmids were found to share an overlapping fragment of 5.6 kb, as could be deduced from their restriction enzyme patterns. Maps of the cosmids share an overlapping fragment of 5.6 kb, as could be deduced from their restriction enzyme patterns. The four stimulating cosmids were found to share an overlapping fragment of 5.6 kb from cosmid 201 in pLAFR3 (49) still stimulate hydrolysis of tributyrin oil after transfer to strain Ps824 (data not shown).

**TABLE 2. Microtiter test results**

<table>
<thead>
<tr>
<th>Cosmid</th>
<th>Lipase productivity of strain with indicated cosmids in²:</th>
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<tbody>
<tr>
<td></td>
<td>Medium 380 + soybean oil</td>
</tr>
<tr>
<td>505</td>
<td>63.5 ± 10.4</td>
</tr>
<tr>
<td>71</td>
<td>40.3 ± 11.4</td>
</tr>
<tr>
<td>201</td>
<td>39.0 ± 5.6</td>
</tr>
<tr>
<td>726</td>
<td>36.8 ± 4.5</td>
</tr>
<tr>
<td>pLAFR3</td>
<td>20.8 ± 2.1</td>
</tr>
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- Lipase productivity is the size of the clarification zone (mm²) on tributyrin oil agar plate/optical density at 600 nm for the 4 selected cosmid strains from the 20 lipase-stimulating strains chosen after microtiter assay done in quadruplicate.

**FIG. 1.** Lipase-enhancing cosmids were selected on the basis of the halo size around a colony on a plate with tributyrin oil (1% [vol/vol]). The halo size is a measure of the amount of lipase activity made by this colony. Strains: Ps1042, with cosmid 201 in Ps537; Ps1049, carrying the subcloned 4.5-kb EcoRI-HindIII fragment of cosmid 201 in pLAFR3; and Ps1029, with pLAFR3 in Ps537.
regulatory proteins a conserved cysteine, whereas NtrC proteins have a methionine (Fig. 3A). The well-conserved R3 region consisting of two \( \alpha \)-helices separated by a turn (34) is clearly identifiable in LipR, and this is one of most important characteristic regions for NtrC-like proteins. The other regions (R5 and R7 [Fig. 3B]) in LipR are fairly homologous to NtrC-like proteins. LipR has a helix-turn-helix motif in the C-terminal part of the protein, like all other NtrC-related proteins. Chou-Fasman (6) secondary structure prediction gives the turn at exactly the same position as for the NtrC proteins, but the lengths of the two \( \alpha \)-helices differ from those for NtrC proteins. The predicted first \( \alpha \)-helix of the helix-turn-helix from LipR is longer than for NtrC proteins. Interestingly, the CbrB protein from \( P. \) aeruginosa (478 residues; accession no. BAB20867) shows 87% identity to LipR (471 residues). The seven well-conserved internal regions and the C-terminal helix-turn-helix are present in CbrB as well. The proteins LipR and CbrB have a high variability only in two regions, where CbrB also has the following additional amino acids compared to LipR: residues 114 to 144 and residues 397 to 414.

Furthermore, Fig. 3A presents a partial alignment of LipQ protein with other NtrC homologues, including their highly conserved histidine kinase A domain (dimerization and phosphoacceptor domain) region; the phosphorylated histidine characteristic for this type of kinase is marked. The N terminus of LipQ up to approximately 450 amino acids corresponds to transmembrane stretches that are followed by a PAS domain (62) (signaling sensor domain) that goes from 632 to 700 in the amino acid sequence. When comparing CbrA (accession no.
BAB20866) with LipQ, the similarity is evident from the lengths (CbrA, 983 residues; LipQ, 984 residues) and the high amino acid identity (79%). In contrast to other NtrB homologues in our alignment, CbrA and LipQ display strikingly long N-terminal sequences (about 600 amino acids longer) corresponding to transmembrane domains. At the beginning of the transmitter domain at position 766 in LipQ (position 766 in CbrA), a conserved histidine can be found within a conserved region (Fig. 3A). This histidine is extremely conserved in prokaryotic sensor transduction histidine kinases, and it represents the autophosphorylation site. The overall identity of the transmitter domain of LipQ in comparison with that of NtrB proteins is moderate; however, the similarity of functionally important residues and regions is apparent (Fig. 3A).

Overexpression of lipQR enhances lipase expression in P. alcaligenes during fermentation. An additional observation supporting the conclusion that LipQR acts as the regulator for lipase expression in P. alcaligenes comes from fermentation studies. The overexpression of lipQR (in strains Ps1039, Ps1040, Ps1041, and Ps1042) results in a large halo on a tributyrin oil plate compared to what is seen for strain Ps537 with empty vector pLAFR3 (49). As an example, the halo formation of strain Ps1042 is shown in Fig. 1.

In a mimicked large-scale fermentation (at a 10-liter scale), strain Ps1039 containing cosmid 505 with LipQR was compared with host strain Ps537 containing the empty vector. A fed-batch fermentation was performed according to the protocol described previously with a batch medium based on citric acid and a feed phase based on soybean oil (13). The lipase yield data plotted against the fermentation time are shown in Fig. 4. The best fit of the data was found with a polynomial (third-order) trend line. The trend line was calculated within an $R^2$ of 0.9858 for the Ps537 curve and an $R^2$ of 0.9934 for the Ps1039 curve. As can be seen from Fig. 4, the lipase production by Ps1039 not only starts at a higher rate but also extends to a level that is threefold higher than that seen for the control. It was verified that during fermentation no loss of the cosmid vector occurred. The other LipQR-overproducing strains

FIG. 3. Sequence alignment of the deduced amino acid sequences of LipQ and LipR with different homologues of two-component systems. The Clustal method with percent accepted mutation residue weight table (Lasergene technology) has been used for the alignment. Dark-shaded residues represent the amino acids that are identical in all aligned proteins. (A) The partial alignment of LipQ from P. alcaligenes M1 (LipQPAL [this study]) with NtrB proteins of Azospirillum brasilense (NtrB3AB [28]), Salmonella enterica serovar Typhimurium (NtrB3ST [25]), Escherichia coli (NtrB3EC [31]), and Pseudomonas aeruginosa (CbrAPAO [37]) is displayed. The presented part of the sequences refers to the highly conserved His kinase A domain, including the position of the autophosphorylation site, which is indicated with an asterisk. (B) The alignment of LipR from P. alcaligenes M1 (LipRPAL [this study]) with NtrC proteins of Azospirillum brasilense (NtrC3AB [28]), Salmonella enterica serovar typhimurium (NtrC3ST [25]), and Pseudomonas aeruginosa (CbrAPAO [37]) is demonstrated. The conserved regions (R1 to R7) that contain the most common features of two-component regulators as discussed in the text are displayed below the alignments. The leftmost numbers correspond to amino acid numbers.

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Inactivation of the lipR gene down-regulates lipase expression. In order to investigate the effects of chromosomal lipR inactivation, we constructed a lipR-negative strain by insertion inactivation, and amplification with Pwo polymerase was conducted on an internal part of lipR by use of forward and backward primers as described in Materials and Methods. Tetrazycline (5 mg/liter)-resistant colonies were checked for the presence of the integration unit by use of Southern hybridization with the PCR fragment as a probe (data not shown). One selected strain was used in further experiments; it was named Ps1100. The lipR-inactivated strain, Ps1100, was tested for lipase expression on a tributyrin oil plate and found to give a halo size reduced from that seen for the Ps93 strain (Fig. 5A). The reduced clearing zone present for the Ps1100 strain could be the result of low basal levels of lipase transcription in the absence of LipR and/or the result of resident esterase activity. Further, to verify the expression levels of the lipA gene for two P. alcaligenes strains, we collected bacterial cells from the agar plates supplemented with tributyrin oil. Figure 5A represents the plate with colonies collected for RNA isolation. Gene expression, quantified by the qRT-PCR method and the comparative critical threshold (ΔΔCT) method, revealed that inactivation of the lipR gene (Ps1100 strain) significantly decreased lipase mRNA levels (Fig. 5B). The relative expression level of the lipA gene was normalized to 16S rRNA, and the level of transcripts was determined relative to that of the Ps93 strain. The Ps1100 mutant gave a relative lipase expression level of 0.03 relative to an assigned arbitrary quantity of 1 for the Ps93 strain (Fig. 5B). These data support our findings that lipase transcript is down-regulated in the absence of LipR protein.

DNA binding activity in crude protein extracts from P. alcaligenes. To test the binding properties of cell extracts from different Pseudomonas alcaligenes strains (Ps537, Ps1039, and Ps1100) to the lipase promoter region P lipA367, a 367-nucleotide fragment corresponding to the lipase promoter regulatory region from −183 to +184 was amplified by PCR. The DNA probe was used in gel retardation assays with decreasing amounts of protein cell extracts. DNA band shifts indicating a protein-DNA complex formation were detected, as shown in Fig. 6. Figure 6A, lanes 1 to 4, presents the mobility of the DNA fragments (P lipA367 and P lipA199) amplified by PCR, corresponding to the lipase promoter and its UAS, which were radioactively end labeled. They were then incubated with decreasing amounts of protein samples. Finally, the mixtures were analyzed by electrophoresis on a 5% nondenaturing polyacrylamide gel (5% TBE gel; Bio-Rad). Top and bottom arrows indicate retarded and nonretarded bands, respectively. (A) P lipA367 probe with/without cell-free protein extracts from Pseudomonas strains at concentrations ranging from 10 μg to 2.5 μg. Lanes: 1, no protein extract added; 2 to 4, probe samples incubated with proteins from the Ps537 strain; 5 to 7, with proteins from Ps1039 strain; 8 to 10, with proteins from Ps1100 strain. (B) P lipA199 probe with/without purified protein samples from E. coli strains at concentrations of 10 μg and 5 μg. Lanes: 1, no protein added; 2 and 3, purified proteins from the DH10B/pME6032LipR strain; 4 and 5, with purified proteins from the DH10B strain.
lipase promoter region P_{lipA367} upon incubation with 0 μg, 10 μg, 5 μg, and 2.5 μg of P_{5537} crude protein extract, respectively. The amount of shifted fragment decreased proportionally with a decreasing amount of the crude protein extract that was added to the assay mixture. A similar observation was made when crude protein extract from the P_{1039} lipR overexpression strain (with cosmid 505) was used in gel retardation (Fig. 6A, lanes 5, 6, and 7). In all cases, the retarded complex migrated to the same position. However, the amount of the DNA probe shifted upon incubation with cell extract of LipR-overproducing strain P_{1039} was higher than for P_{5537} cell extract.

Surprisingly, the gel shift assay with a cell-free protein extract from the P_{1100} strain still shows some binding to lipase promoter sequence P_{lipA367}. Lanes 8 to 10 of Fig. 6A present the mobility of the lipase promoter after incubation with 10 μg, 5 μg, and 2.5 μg of P_{1100} crude protein extract, respectively. As can be observed by the presence of retarded bands, the binding increased proportionally with increasing concentrations of proteins from cell extract. Overall, however, the intensity of the retarded band is low compared with what is seen for the strains carrying LipR protein (P_{5537} and P_{1039}).

**Gel retardation with partially purified LipR from E. coli demonstrates binding to the lipase promoter.** To support the argument that LipR protein interacts with the lipase promoter, we partially purified the protein from an E. coli DH10B overexpressing strain and tested it in a gel retardation assay. Conditions of expression are described in Materials and Methods. SDS-PAGE analysis and peptide mass mapping allowed the identification of a 51.9-kDa protein as a product of lipR gene. Cell extracts from E. coli DH10B strains (lipR mutant and lipR⁻ strains) were used for one-step purification on a heparin-Sepharose column. LipR (E. coli DH10B/pME6032LipR) present in a cell-free protein extract was found to bind effectively to the heparin matrix at pH 6. Peak fractions, analyzed by SDS-PAGE (with approximately 50% LipR purity), were taken to investigate their abilities to bind to radiolabeled DNA probe P_{lipA199} by gel retardation assay. P_{lipA199} corresponds to the lipase promoter regulatory region from −183 to +16 (7). This shorter probe was created, as the P_{lipA367} probe used initially had the tendency to form dimers (data not shown). In EMSA, we compared the binding abilities of fractions originating from LipR⁺ and LipR⁻ E. coli extracts eluted from the heparin column. Figure 6B demonstrates a band shift indicating a formation of a DNA-protein complex for a fraction containing LipR protein (lanes 2 and 3), whereas no such complex can be seen for a fraction lacking LipR (lanes 4 and 5).

**DISCUSSION**

The present studies show for the first time that a two-component system, named LipQ-LipR, is involved in the regulation of lipase expression. The transcription start of the lipase operon in P. alcaligenes is similar to the consensus of σ54-type promoters with a UAS (7). This points to a mechanism of positive control of lipase gene transcription by a regulatory protein similar to the one found for other σ54 promoters (30). In searching for such a regulatory protein, we first embarked on an attempt to complement lipase-negative mutants in *Pseudomonas alcaligenes*. However, the number of lipase-negative mutants found was far higher than expected. Most likely, many of these mutants were not deficient in export or protein-folding functions, as found for *P. aeruginosa* (11). In order to overcome this problem, we used a selection for positive phenotypes—the phenotype enhancement method—to search for elements that are in a direct way controlling lipase production (14). Thus, a cosmid library was introduced into *P. alcaligenes* strain P_s770, which carries a pJRDLipAB plasmid for increased lipase production. It was anticipated that the selection of lipase-hyperproducing strains would result in the identification of factors limiting lipase expression. From three independent tests with 485 strains containing the introduced cosmid library, which covers about four times the size of the chromosome, 20 were selected with hyperproduction of lipase, as judged by halo formation on a tributyrin oil plate. One of these selected 20 hyperproducing strains was identified as carrying the complete *P. alcaligenes* type II secretion pathway described earlier (14).

During fermentation experiments, due to plasmid loss, tetracycline and/or neomycin resistance for some of the 20 tested strains was gone. Therefore, the cosmids were introduced independently in strain P_{5537} containing a single chromosomal lipase operon, lipAB. Four cosmid-containing strains with bigger halos on tributyrin oil plates were found to share an identical DNA fragment that has similarity with the family of two-component regulator systems represented by NtrBC (34). Comparison and alignment of data reveal that the first gene encoding LipQ protein is similar to that for the NtrB type of kinase and the second gene encoding LipR protein resembles that for the NtrC type of DNA binding regulator protein. In addition, relaxation of control by overexpression of the two-component system resulted in a threefold increase of lipase production in a mimicked large-scale fermentation. The enhanced lipase production fits perfectly with the copy number of plARF3 (49) derivates in *P. alcaligenes* (two to four copies) (13).

As mentioned earlier, the LipRQ system bears resemblance to the CbrAB two-component system from *P. aeruginosa*. Importantly, CbrB and LipR show significant amino acid sequence similarities, and the same is true for CbrA and LipQ. Yet, it is interesting that in contrast to LipR, the homologous CbrB protein so far has not been shown or indicated to share the same mode of action on the lipase promoter. The CbrAB two-component system of *P. aeruginosa* was proposed to be involved in the utilization of carbon and nitrogen sources (37, 51), thus playing an important role in environmental adaptation. Still, there are no reports yet implying the direct interaction of any promoter sequences with CbrB transcriptional activator, and the histidine operon and/or arginine operon are potential candidates only. By inspecting the promoter sequences of these two operons, the probable σ54 box (−12 to −24) can be found just for the arginine operon, with no obvious σ54-type promoter for the histidine operon (51). Interestingly, existing reports indicate that CbrB appears to be important in the expression of the histidine operon in particular (37, 44, 51). In contrast to the CbrAB system, the LipQR system has not been shown to be essential in carbon-nitrogen utilization in *P. alcaligenes*, indicating that the LipQR and CbrAB regulatory systems may play different regulatory roles under
physiological conditions. Moreover, the signals that determine the CbrAB activity remain unknown, and regulation by the CbrAB system seems to be a complex mechanism that includes the regulation of several catabolic pathways. Moreover, it is not known yet whether lipQR genes are cotranscribed.

Our results demonstrate that the overexpression of lipQR up-regulates (Fig. 2) and the inactivation of lipQR down-regulates (Fig. 5) lipase expression. Moreover, the upstream region of the lipase gene from P. alcaligenes possesses, at the expected distance from a promoter at –24 to –12, a UAS (TGTT-N11-ACA) (3) which shows all the attributes to serve as a template for the binding of the activated LipR regulator. Thus, the involvement of LipR in the regulation of lipase expression seen from fermentation studies, coupled with the demonstrated down-regulation of lipase at the transcriptional level by inactivation of lipQR (qRT-PCR assay) (Fig. 5B), substantiates our hypothesis and establishes LipR as a lipase regulator.

Finally, results from the band shift experiment (P_{Ps1039}) with partly purified proteins from E. coli lipR+ versus E. coli lipR− negatives demonstrate LipR protein DNA binding properties. The retardation of the DNA probe by formation of the DNA-LipR complex was present only for a protein sample containing LipR (Fig. 6B). Supporting results come also from EMSA with Ps537 and Ps1039 cell crude protein extracts (Fig. 6A, lanes 2 to 7), where more DNA formed a complex with proteins from Ps1039 than with proteins from the Ps537 strain, which is in agreement with the fact that Ps1039 overexpresses LipR. The presence of retarded bands with cell-free protein extract from the Ps1100 strain (lipR mutant) (Fig. 6A, lanes 8 to 10) most likely is the result of binding other protein(s). The lipase promoter sequence includes a long DNA stretch with a UAS up to the region from other protein(s). The lipase promoter sequence includes a long DNA stretch with a UAS up to the region from other protein(s). The lipase promoter sequence includes a long DNA stretch with a UAS up to the region from other protein(s). The lipase promoter sequence includes a long DNA stretch with a UAS up to the region from other protein(s).

In conclusion, we have found and cloned a new two-component system, lipQR, which is involved in the regulation of lipase expression in P. alcaligenes, with LipQ being the putative phosphorylating component and LipR the DNA binding protein. The commercial relevance of this finding is exemplified in the hyperproduction of lipase upon overproduction of LipR.

ACKNOWLEDGMENTS

This research was partly sponsored by EU grants BIO4-CT96-00119 and QLK3-CT-2002-02086.

We thank Lydia Dankmeyer and Ronald Hommes for experimental help.

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