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Pseudomonas as a microbial enzyme factory

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**LipR, a response regulator, directly controls the
lipase gene expression in *Pseudomonas*
*alcaligenes***

Joanna Krzeslak, Ronald van Merkerk, Robbert H. Cool, and Wim J. Quax

Manuscript in preparation

LipR, a response regulator, directly controls the lipase gene expression in *Pseudomonas alcaligenes*

Previous work has demonstrated that the expression of the *Pseudomonas alcaligenes lipA* gene, which encodes an extracellular lipase, is governed by the LipQR two-component system, with LipR being the DNA binding protein. This report demonstrates that LipR binds directly to the lipase promoter sequence as monitored by Surface Plasmon Resonance studies. Moreover, it is revealed that LipR phosphorylation is essential for specific binding of LipR to a DNA fragment comprising the lipase promoter. In addition, we show that LipR exhibits an intrinsic ATPase activity that can be stimulated by the presence of DNA and by LipR phosphorylation. Finally, the abolished production of β -galactosidase from the *lipA-lacZ* transcriptional fusion in *lipR* and *rpoN* mutant strains, confirms the involvement of LipR and RpoN in the lipase expression regulation cascade.

Introduction

Bacteria often experience "life on the edge" as they confront severely changing environmental cues to which they must respond in a quick and accurate manner. For this purpose, they evolved the environmental adaptation strategies, such as modulation of the expression of specific genes facilitated by sophisticated transduction systems, called two-component systems¹⁻⁴. Two-component systems are often involved in signal transduction processes during sporulation, chemotaxis, and virulence. Typically, these systems comprise of two proteins, an integral membrane sensor kinase and a cytoplasmic response regulator. These partners communicate by reversible phosphorylation in response to environmental stimuli. The response regulator, depending on its phosphorylation state, activates or represses gene transcription by binding to distinct DNA sequences on the genome. This phenomenon ultimately leads to an optimal adaptation of bacteria to their ecological niches.

Pseudomonas alcaligenes is a non-pathogenic Gram-negative bacterium that can secrete commercially relevant enzymes, such as lipases and proteases. These enzymes can be easily recovered from the extracellular medium at high quantities⁵. Lipases (triacylglycerol acylhydrolase EC 3.1.1.3) play an important role among biocatalysts, as they catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids⁶. Their potential and industrial value is reflected in a broad spectrum of biotechnological applications, such as household detergents, processing of fats, and synthesis of pharmaceuticals⁶. The need for effective and efficient lipases with desired properties and industrial potential has drawn a lot attention towards, in particular, lipases from Pseudomonads. In general, expression of microbial lipases can be influenced by a number of environmental factors such as temperature, pH, nitrogen, carbon, lipid sources, and dissolved oxygen concentration⁷⁻⁹. The presence of lipids and detergents has been shown to positively stimulate lipase production¹⁰. For *P. alcaligenes*, increased production of lipase was observed when cultures were grown in soybean oil-enriched medium^{5,11}. However, the definite molecular mechanism underlying the regulation of the lipase gene expression is yet to be elucidated.

For lipase expression, Pseudomonads like *P. aeruginosa* appoint two genes lying in one operon, where a *lipA* gene is followed by a *lipB* gene (known also as *lif* or *lipH*). The first one

encodes the extracellular lipase protein and the second one encodes the helper gene involved in lipase periplasmic folding¹²⁻¹⁴. The promoter sequence of the *lipA* gene and its UAS (upstream activating sequence) in *P. alcaligenes* were characterized by Cox *et al.*¹⁵. Recently, we have identified a two-component regulatory system, LipQR, in *P. alcaligenes* to be involved in the lipase expression regulation¹⁶.

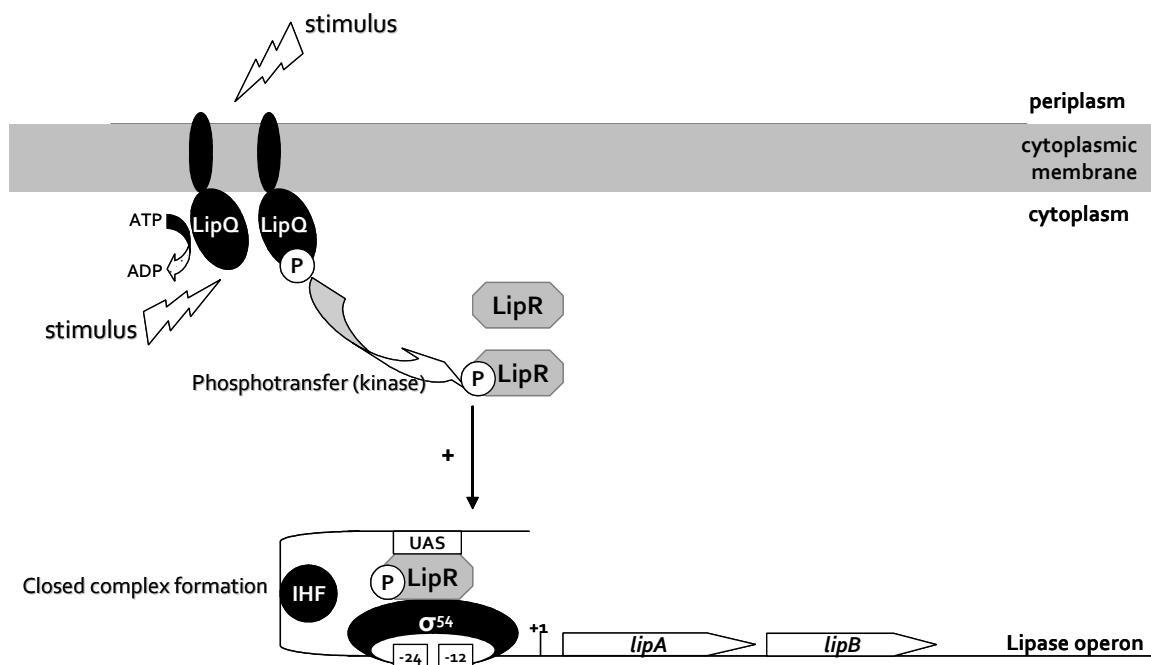


Figure 1. Hypothetical model depicting the mechanism of the transcriptional activation of the lipase *lipAB* operon by the LipQR two-component regulatory system. The sensor component is a histidine protein kinase, LipQ, embedded in the cellular membrane. The N-terminal domain of this protein kinase functions as the input region, detecting environmental stimuli and undergoing autophosphorylation. Activate LipQ phosphorylates the LipR response regulator that in turn interacts with the upstream activating sequence (UAS) of the promoter (-12/-24). Firstly, σ^{54} binds to the promoter in the absence of core enzyme and recruits the core to form a stable closed complex. Open complex formation occurs when the RNA polymerase is activated by the LipR response regulator (enhancer protein) which binds to the UAS. In most cases this activation is mediated by bending of the DNA generated by the integration host factor (IHF) binding to the sequence between promoter and UAS. The shape and volume of the different proteins are symbolic.

Proteins transcribed from the *lipQR* operon show similarity with the members of the NtrBC-family of two-component regulatory proteins. LipQ, as a putative sensor histidine kinase, is thought to sense changes in environmental factors that regulate autophosphorylation. Autophosphorylation subsequently leads to activation of its cognate response regulator, LipR, by transfer of the phosphate group to the conserved aspartate residue at position 52. By direct binding to the lipase promoter, phosphorylated LipR, LipR-P, in concert with the transcription factor RpoN and RNA polymerase is thought to initiate transcription of the lipase gene (Figure 1). The *rpoN* gene encodes an alternative sigma factor,

RpoN (σ^{54}), which is widely distributed among Gram-negative bacteria. Generally, RpoN is not required for survival and the RpoN-dependent transcription may be important in utilization of various nitrogen and carbon sources, energy metabolism¹⁷, RNA modification¹⁸, chemotaxis, development, flagellation, electron transport, response to heat and phage shock¹⁹, and expression of alternative sigma (σ) factors²⁰. The response regulators that act in concert with the RpoN-RNA polymerase to form an open complex at the promoter sites are equipped with ATPase domains of the AAA⁺ class^{21,22}. The conformational change of response regulators, triggered by phosphorylation of an aspartate residue in the regulatory domain, induces the oligomerization of the central ATPase domain. Oligomerization appears to be important for ATP hydrolysis and productive interaction with a σ^{54} -holoenzyme²³⁻²⁵.

We have previously shown that LipR is involved in regulation of the lipase gene expression and that a specific DNA binding activity is present in cell-free protein extracts from *P. alcaligenes*¹⁶. As a continuation of that study, we report here a direct specific binding of the transiently phosphorylated LipR protein to the lipase promoter. Furthermore, we demonstrate the ATPase activity of LipR and the involvement of the RpoN factor in lipase expression regulation.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

The plasmids and bacterial strains used in this study are listed in Table 1. *Pseudomonas alcaligenes* and *Escherichia coli* were propagated in LB (10 g/liter Bacto tryptone, 5 g/liter Bacto yeast extract, 10 g/liter NaCl) medium or 2x TY medium (16 g/liter Bacto tryptone, 10 g/liter Bacto yeast extract, 5 g/liter NaCl, pH 7.0) as a liquid or solid medium. For tributyrin plate assay a minimal medium [0.4 g K₂HPO₄, 1.0 g (NH₄)₂SO₄, 0.32 g MgSO₄·7H₂O, 0.25 g CaCl₂·2H₂O, 4.0 mg CoCl₂·6H₂O, 3.2 mg MnSO₄·1H₂O, 6.2 mg FeSO₄·7H₂O, 1.2 mg ZnSO₄·7H₂O, 0.3 mg CuSO₄·5H₂O, 0.3 mg H₃BO₃, 0.3 mg Na₂MoO₄·2H₂O, 0.1 mg KI, 0.6 g citric acid, 47 g HEPES, and 15 g agar per 1 liter, pH 7.5] was used. Antibiotics were used at the following concentrations: tetracycline 5 mg/liter and carbenicillin 100 mg/liter for *P. alcaligenes*, and ampicillin 100 mg/liter and tetracycline 25 mg/liter for *E. coli*.

Construction of plasmids, strains, and DNA probes

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of competent *E. coli* cells were carried out as described by Sambrook *et al.*²⁶. *P. alcaligenes* was transformed as described by Wirth *et al.*²⁷ and modified by Gerritse *et al.*¹¹. The enzymes used for DNA manipulations were purchased from New England BioLabs and applied according to the instructions of the manufacturer. Plasmid and cosmid DNA were isolated using the Qiaprep spin miniprep kit (Qiagen). PCR was carried out with *Phusion* polymerase (Finnzymes) using chromosomal DNA of *P. alcaligenes* as a template or with *Taq* polymerase (Fermentas) to generate DNA probes for binding assays. (i) **Construction of an *rpoN* mutant strain.** In order to create the *rpoN* mutant by insertional inactivation, an internal *rpoN* fragment (bp 298 to 1011 relative to the translation start site) was amplified using two primers: RpoN₂₉₈MutPvuI-F (5' CGA GCG CCG ATC GAA CGA GCT GCA CGG TCG ATA C 3', where the *PvuI* is underlined and the base shown in boldface was introduced to generate a frameshift mutation) and RpoN₁₀₁₁EcoRI-R (5' TGG TTG CGG AAT TCG GTG TTA TCG GCG CTG GAG T 3', where the *EcoRI* site is underlined). A purified and *PvuI*-*EcoRI*-digested internal *rpoN* fragment was introduced into a pBR322 vector of 3,737 bp (*PvuI*-*EcoRI* generated) followed by *E. coli* DH5 α transformation. Tetracycline-resistant (Tet^r) transformants were selected on LB agar plates containing 25 mg/liter tetracycline, and plasmid DNA was isolated, sequenced, and electroporated to *P. alcaligenes* strain Ps93. Integrants were selected on 2x TY plates containing 5 mg/liter tetracycline and were checked by Southern analysis. (ii) **Construction of a *lipA-lacZ* transcriptional fusion.** To construct the *lipA-lacZ* transcriptional fusion, an upstream *lipA* gene fragment of 273-bp comprising of the UAS, the promoter -12 to -24, and the RBS was amplified from the plasmid pJRDlipAB with the LipALacZ-F (5' GAG CTC GAA TTC CCT GGC TGG CAG G 3') and LipALacZ-R (5' GGT TTT CTT AAG CTT CAT

GTT TTG CTC T 3') primers carrying the *EcoRI* and *HindIII* restriction sites (underlined). Next, the *EcoRI-HindIII* fragment was inserted upstream the promoterless-*lacZ* gene in the pTZ110 vector, generating the pTZlipA fusion plasmid (verified by sequencing). (iii) **DNA probes.** The $P_{lipA199}$ probe (lipase promoter sequence) was generated by PCR as described previously¹⁶, the $P_{lipA199}$ -Biotin probe was amplified using the biotinylated forward primer ForLipA-biotin and the backward primer BackLipA2 (sequences are described in ref. 16). The DNA fragment of 197-bps, named here rpoD-Biotin, corresponding to the internal part of the *rpoD* gene from *P. aeruginosa* PAO1 was used as non-specific biotinylated DNA in SRS experiments. This fragment was amplified from chromosomal DNA of *P. aeruginosa* PAO1 by use of the forward ForRpoD-biotin primer (5' GGG CGA AGA AGG AAA TGG TC 3') and the backward BackRpoD primer (5' CAG GTG GCG TAG GTG GAG AA 3'). Purification of PCR products was done by use of the PCR purification kit from Qiagen according to the supplier's instructions.

Table 1. Bacterial strains and plasmids used in the study.

Strains	Description	Reference
<i>P. alcaligenes</i>		
Ps93	Restriction negative, modification positive	5
Ps1100	Restriction negative, modification positive; <i>lipR</i> mutant; Tet ^r	16
Ps1101	Restriction negative, modification positive; <i>rpoN</i> mutant; Tet ^r	This study
<i>E. coli</i>		
DH5 α	F- ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (rk-, mk+) <i>phoA supE44 thi-1 gyrA96 relA1</i> λ -	Invitrogen
DH10B	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 endA1</i> <i>araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU galk</i> λ - <i>rpsL nupG</i>	Invitrogen
Plasmids	Description	Reference
pBR322	Amp ^r , Tet ^r	28
pBR322RpoNtr	pBR322 with an internal <i>rpoN</i> gene fragment; Amp ^r	This study
pTZ110	Cb ^r ; broad-host-range <i>lacZ</i> transcriptional fusion vector	29
pTZlipA	Cb ^r ; pTZ110 with a 273-bp PCR fragment of upstream <i>lipA</i> gene region; including UAS, promoter -12/-24, and RBS	This study
pME6032	Broad-host-range cloning vector, Tet ^r	30
pME6032LipR	<i>lipR</i> -expressing plasmid, Tet ^r	16

Abbreviations: Amp^r, ampicillin resistance; Cb^r, carbenicillin resistance; Tet^r, tetracycline resistance.

Proteins expression, preparation and purification

The Erlenmeyer flask containing 2x TY medium (supplemented with tetracycline at 5 mg/liter, if necessary) was inoculated (100x dilution) with overnight culture of Ps93/pME6032LipR and grown at 20°C for 15 h after induction with 0.5 mM IPTG at the OD₆₀₀ 0.5. Next, the cell culture was harvested by centrifugation. The pellet was resuspended in a lysis buffer consisting of 50 mM Tris-HCl, pH 6.0, 2 mM EDTA, 0.5 mg/ml lysozyme, and 10% glycerol supplemented with Complete Mini protease Inhibitor (Roche), sonicated (output 4, 40% duty cycle on a Sonifier 250; Branson), and the membrane fractions were removed by centrifugation (30 min, 4°C, and 17,000 x g), yielding a cell-free protein extract. (i) **LipR purification.** Overexpressed LipR from *P. alcaligenes* Ps93/pME6032LipR was purified by a three-step purification protocol using the ÄKTA Explorer system (GE Healthcare). Firstly, the cell-free protein extract was subjected to affinity chromatography on a heparin-Sepharose column (GE Healthcare) and eluted with a NaCl gradient (binding buffer: 50 mM sodium phosphate, pH 6.0, 10% glycerol, and 10 mM beta-mercaptoethanol; elution buffer: 50 mM sodium phosphate, pH 6.0, 10% glycerol, 10 mM beta-mercaptoethanol, and 1 M NaCl). Collected fractions were analyzed on a SDS-PAGE gel stained with Coomassie brilliant blue (CBB) and analyzed by Western blotting and immunodetection with LipR antibody. Secondly, selected fractions containing LipR were pooled and loaded on a phenyl-Sepharose column (GE Healthcare). The starter buffer was composed of 50 mM sodium phosphate, pH 8.0, 10% glycerol, 10 mM beta-mercaptoethanol, and 1 M ammonium sulfate, the elution buffer was composed of 50 mM sodium phosphate, pH 8.0, 10% glycerol, 10 mM beta-mercaptoethanol. Proteins were eluted using linear gradient of 1-0

M ammonium sulfate and analyzed by SDS-PAGE, next fractions containing LipR were concentrated (30 kDa MWCO; Vivaspin) and subjected to gel filtration (Superdex 75 HR 10/30 column, buffer containing 50 mM Tris-HCl at pH 8.0, 20 mM NaCl, 10% glycerol, and 10 mM beta-mercaptoethanol). The concentration of proteins was determined by the Bradford method³¹ using bovine serum albumin (BSA) as a protein standard (Pierce). **(ii) Antibody production.** Purified LipR was loaded on a 10% SDS-PAGE gel. The excised piece of SDS-PAGE containing a LipR protein was used for antibody generation (Eurogentec S.A.). **(iii) In vitro phosphorylation of LipR.** The wild type LipR protein (LipR) was phosphorylated (LipR-P) by use of a low weight phosphate donor, carbamoyl phosphate (purchased from Sigma). The reaction was performed at 37°C for 1 h in a buffer consisting of 50 mM Tris-HCl at pH 7.0, 7.5 mM MgCl₂, 1 mM DTT, and 50 mM disodium carbamoyl phosphate. Directly after phosphorylation reaction the LipR-P protein was used in a gel retardation assay, SPR experiment, or ATP hydrolysis assay.

Electrophoretic mobility shift assay (EMSA)

A gel retardation assay, with purified LipR (from the Ps93/pME6032LipR strain), was carried out as described by Ebbole and Zalkin³². The DNA probe, P_{lipA199}, was radioactively end labeled with the T₄ polynucleotide kinase by use of [γ -³²P]ATP. The protein fractions and a probe were premixed on ice in a binding buffer [20 mM Tris-HCl at pH 8.0, 20 mM NaCl, 5 mM CaCl₂, 0.5 mM DTT, 1 mM EDTA, 10% glycerol, 10 mg/liter poly(dI-dC), and 0.1 g/liter BSA]. After 20 min at 37°C, samples were loaded on a nondenaturing gel (5% Tris-borate-EDTA [TBE] gel; BioRad). Gels were run at 100 V in 1x TBE buffer (89 mM Tris, 89 mM boric acid, and 2.8 mM EDTA; solution at pH 8.3), dried, and autoradiographed.

Surface plasmon resonance (SPR)

SPR measurements were performed on a BIAcore 3000 using a streptavidin-coated SA sensor chip (GE Healthcare). Each surface of the chip was conditioned as recommended by the manufacturer, and equilibrated with HBSN buffer (GE Healthcare; 10 mM HEPES, pH 7.4, 150 mM NaCl). A volume of 260 μ l of 0.6 μ g/ml biotinylated DNA fragments, specific P_{lipA199}-Biotin or nonspecific rpoD-Biotin, were injected at a flow rate of 5 μ l min⁻¹ across a streptavidin sensor chip and immobilized resulting in 700 resonance units (RUs). One flow cell was left unmodified as the control. Protein binding experiments were performed at 25°C at a flow rate of 70 μ l min⁻¹. Phosphorylated (LipR-P) and wild type LipR (LipR) were diluted in HBSN buffer to 370 nM prior injections. The sensor surface was regenerated after each cycle with 3 M MgCl₂ and 30 s contact time.

ATPase assay

A standard ATPase assay was performed in a Tris buffer (50 mM Tris-HCl, pH 7.0 and 5 mM MgCl₂) at 37°C in a final reaction volume of 50 μ l. Phosphorylation was achieved by preincubation of LipR at 37°C for 1 h with carbamoyl phosphate. Reactions were initiated by addition of unlabelled ATP mixed with 1.6 mM [γ -³²P]ATP (6000 Ci/mmol). Incubations were at 37°C for 40 min. The LipR protein was added to a final concentration of either 125 nM, 250 nM, or 500 nM, as indicated in the figure legends. The DNA fragment, P_{lipA199}, corresponding to the lipase promoter was present at concentrations of 15 nM, 30 nM, or 60 nM. The reactions were terminated by addition of a suspension of 5 % (w/v) activated charcoal in 1 M HCl that absorbs proteins and nucleotides, but not inorganic phosphate³³. The samples were centrifuged (2 min, 13,000 rpm, 4°C), thereafter the supernatant was quickly but carefully transferred to another tube. This step was repeated to ascertain complete removal of the charcoal. The amount of ³²P_i released was quantified by liquid scintillation counting.

Tributyryl plate assay

P. alcaligenes strains were grown overnight in 2x TY liquid medium, next the OD₆₀₀ was determined and the same amount of cells of each culture, bring to 5 μ l, was spotted on tributyrin oil (1%, v/v) agar plates amended with tetracycline, if necessary. Plates were incubated at 37°C for 48 h.

β -galactosidase assay

Lipase promoter activity in *P. alcaligenes* was analyzed according to the previously described method³⁴. Briefly, overnight cultures of *P. alcaligenes* (Ps93, Ps1100, and Ps1101) harboring the *lipA-lacZ* fusion plasmid, pTZlipA, or the pTZ110 plasmid (negative control) were diluted in fresh 2x TY medium and grown at 37°C. Samples were harvested at different intervals, as indicated at the figure legend, for absorbance readings at 600 nm and β -

galactosidase activity determinations. The growth medium, for strains carrying pTZlipA or pTZ110, was amended with carbenicillin (100 mg/liter) and for the *lipR* and *rpoN* mutant strains also with tetracycline (5 mg/liter). Cells were permeabilized with CHCl_3 and sodium dodecyl sulfate. The results shown are representative of at least two independent experiments. The variance is indicated by error bars. Samples from the Ps93, Ps1100, and Ps1101 strains did not show any β -galactosidase activity, whereas strains harboring the promoterless pTZ110 vector revealed background activity that was deducted from the β -galactosidase activities gained from the strains carrying the *lipA-lacZ* fusion.

Results

Purification of LipR

In order to perform DNA binding studies, the response regulator, LipR, was purified from *P. alcaligenes* by a three-step chromatography. Briefly, the Ps93 strain, carrying the pME6032LipR plasmid encoding the *lipR* gene, was grown in 2x TY medium for 15 h at 20°C after addition of IPTG (0.5 mM). Bacterial cells were harvested, lysed by sonication, and subjected to the first purification step on a heparin-Sepharose column. Optimal binding of LipR to the heparin matrix was observed when the lysis buffer and the binding buffer were adjusted to pH 6.0. Peak fractions, containing LipR (with approximately 50% purity), were pooled, amended with ammonium sulfate, and loaded on a phenyl-Sepharose column. This purification step was followed by the last step on a gel filtration column. Finally, as judged from SDS-PAGE analysis, the LipR protein was recovered up to <95% purity. Figure 2A presents a detection of purified LipR by Coomassie brilliant blue SDS-PAGE (lane 1) and immunodetection with the LipR antibody (lane 2).

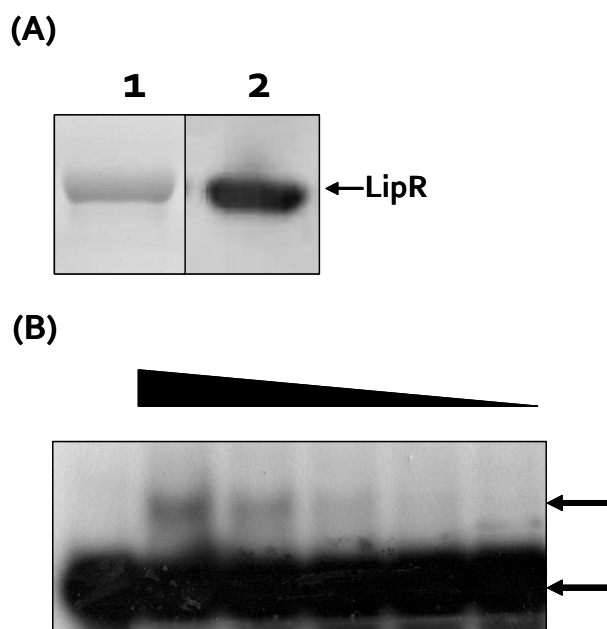


Figure 2. (A) Detection of purified LipR protein. The purified LipR protein was visualized by CBB stained SDS-PAGE gel (lane 1), and Western blotting with immunodetection using a LipR antibody (lane 2). The LipR protein was purified from a cell-free protein extract of Ps93/pME6032LipR in a three-step chromatography strategy using a heparin-Sepharose, a phenyl-Sepharose, and gel filtration column. (B) Gel retardation with purified LipR. A $P_{lipA-199}$ DNA fragment, amplified by PCR, corresponding to the lipase promoter and its UAS, was radioactively end labeled. It was then incubated with decreasing amounts of protein samples. Finally, the mixtures were analyzed by electrophoresis on a 5% nondenaturing polyacrylamide gel. Top and bottom arrows indicate retarded and nonretarded bands, respectively. The big triangle indicates a decrease in LipR concentration of 2-fold per lane, ranging from 1 μM to 62.5 nM.

Binding of LipR to the lipase promoter

To examine the binding properties of LipR to the lipase promoter region, a 199 nucleotide fragment ($P_{lipA199}$) including the lipase promoter and UAS spanning from -183 to +16¹⁵ was amplified by PCR. This fragment was used in gel retardation assays with different amounts of LipR. Figure 2B shows the mobility of $P_{lipA199}$ probe upon incubation with decreasing amounts of purified LipR. The amount of shifted fragment decreased proportionally with a decreasing amount of the LipR protein that was added to the assay mixture. To investigate the effect of LipR phosphorylation on its DNA binding properties, we used *in vitro* phosphorylated LipR-P in the binding assay. However, in this assay phosphorylation did not result in enhanced LipR binding activity to the lipase promoter (data not shown).

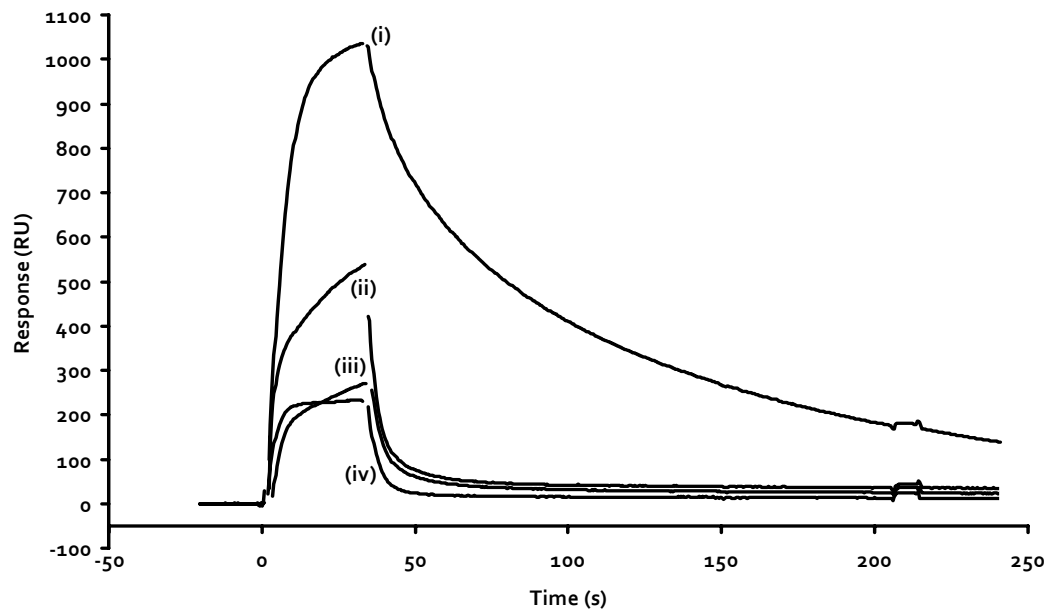


Figure 3. Time versus response SPR sensogram. The biotinylated DNA fragments corresponding to the lipase promoter ($P_{lipA199}$ -Biotin) or the nonspecific DNA sequence ($rpoD$ -Biotin) were immobilized on a streptavidin sensor chip. The wild type LipR or LipR-P (phosphorylated) diluted to 370 nM in HBSN buffer were injected for 30 s (association phase). This was followed by injection of the HBSN buffer alone (dissociation phase). The graph shows the binding of: (i) LipR-P to $P_{lipA199}$ -Biotin, (ii) LipR to $P_{lipA199}$ -Biotin, (iii) LipR to $rpoD$ -Biotin, and (iv) LipR-P to $rpoD$ -Biotin.

Phosphorylation is essential for specific binding of LipR to the lipase promoter

The binding of the wild type LipR and phosphorylated LipR-P to the lipase promoter was assessed in real-time by use of surface plasmon resonance (SPR). Biotinylated DNA fragments containing the lipase promoter ($P_{lipA199}$ -Biotin) or the nonspecific sequence ($rpoD$ -Biotin) were immobilized on a streptavidin sensor chip, and 40 μ l of 370 nM LipR or carbamoyl

phosphorylated LipR-P was injected over the DNA surface at a flow rate of $70 \mu\text{l min}^{-1}$. LipR binds slightly better to the immobilized $P_{\text{lipA}199}$ than to rpoD , but in both cases the binding is not very strong as demonstrated by the high dissociation rate. Phosphorylation does not greatly affect the binding to rpoD , but results in an enormous increase in binding to $P_{\text{lipA}199}$ as demonstrated by the higher response and the slower dissociation (Figure 3). Thus, phosphorylation of LipR with carbamoyl phosphate stimulates an efficient and specific LipR-DNA interaction.

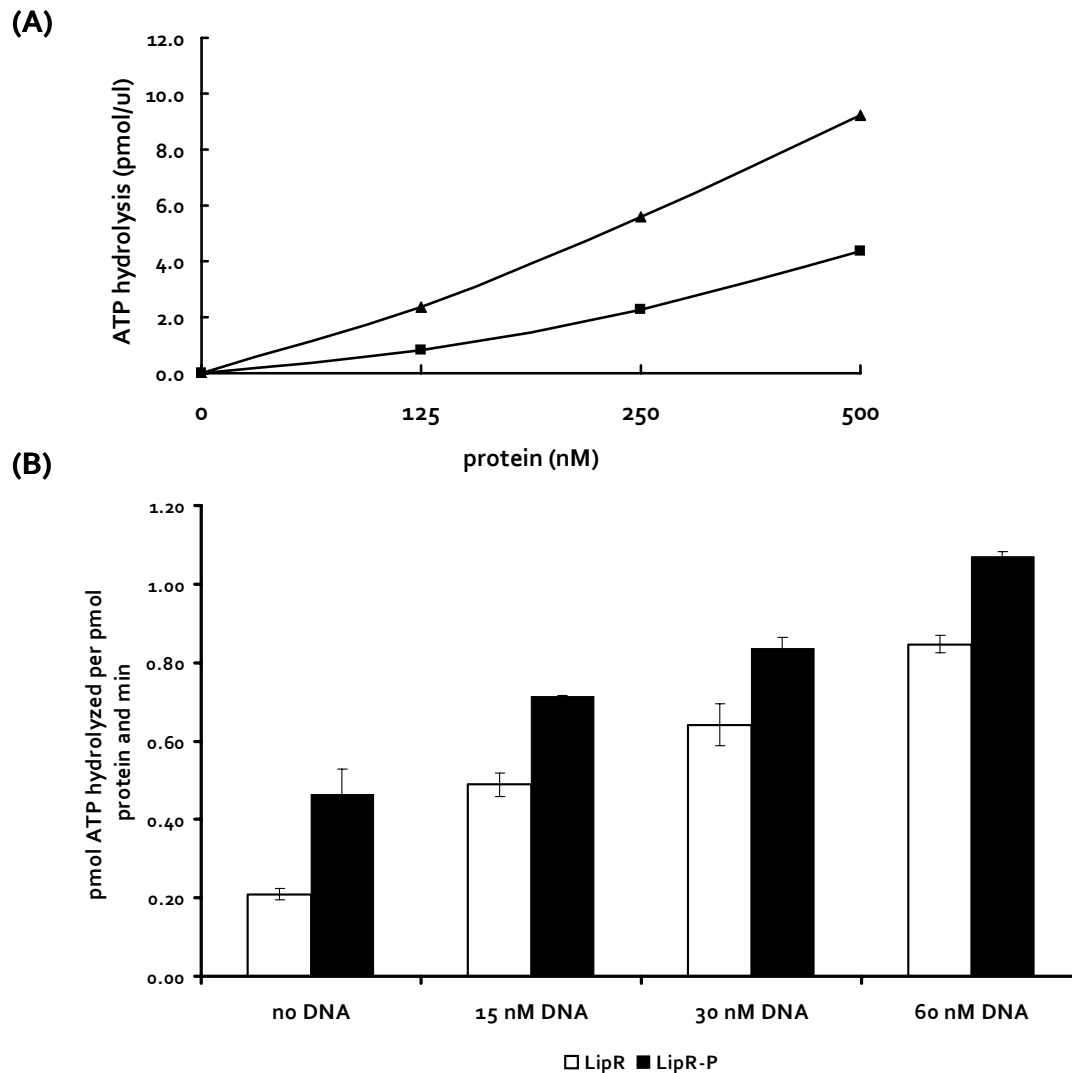


Figure 4. ATPase activity. Reactions (final vol $50 \mu\text{l}$) with wild type LipR or LipR-P (phosphorylated) were incubated as described in the methods section for 40 min at 37°C . Hydrolysis was initiated by addition of $1.6 \text{ mM } [\lambda\text{-}^{32}\text{P}]\text{ATP}$ to a specific activity of 56 cpm/pmol . **(A)** Comparison of the ATPase activities of LipR and LipR-P. The reactions contained the indicated amounts (125 nM , 250 nM , and 500 nM) of LipR (square symbols) or LipR-P (triangles). **(B)** ATPase activity of LipR and LipR-P in the presence of DNA. LipR (500 nM) or LipR-P (500 nM) were incubated with a 199-bp DNA fragment ($P_{\text{lipA}199}$), at the following concentrations 15 nM , 30 nM , or 60 nM .

LipR has an ATPase activity stimulated by phosphorylation and by DNA presence

To test the activity of LipR we performed an ATPase assay. For this assay, we examined the ATP hydrolysis by wild type LipR (LipR) and LipR phosphorylated with carbamoyl phosphate (LipR-P) at different protein concentrations. Figure 4A shows that LipR-P displays 2.5-fold increased ATPase activity, in comparison to that of the wild type LipR protein. The increasing protein concentration of either LipR or LipR-P resulted in higher total ATP hydrolysis (Figure 4A). Yet, the rate of ATP hydrolysis expressed as pmol ATP hydrolyzed/min/pmol of protein (LipR or LipR-P) was alike at different protein concentrations. The rate of ATP hydrolysis by wild type LipR at 125 nM, 250 nM, and 500 nM concentrations was between 0.17 - 0.22 pmol/min/pmol LipR. The LipR-P protein at 125 nM, 250 nM, and 500 nM concentrations hydrolyzed ATP with rates between 0.46 - 0.55 pmol/min/pmol LipR-P. This demonstrates that LipR ATPase activity is stimulated by phosphorylation but not protein concentration. Further, we have observed that the ATPase activity of LipR and LipR-P was stimulated in the presence of DNA. For this experiment, we incubated 500 nM LipR or LipR-P proteins with increasing amounts (15 nM, 30 nM, 60 nM) of a P_{lipA199} DNA fragment. As can be seen on a Figure 4B, addition of DNA stimulated the ATPase activity of both LipR and LipR-P. Yet, the greater degree of stimulation was observed for the LipR protein (4-fold at 60 nM DNA concentration versus a sample without DNA) in comparison to LipR-P (2.4-fold at 60 nM DNA concentration versus a sample without DNA). Overall, the highest stimulation was observed at the 60 nM DNA concentration. Finally, the highest ATP hydrolysis rate (1.07 pmol/min/pmol protein) was recorded for the reaction with 500 nM LipR-P and 60 nM DNA (Figure 4B), which is 5-fold higher than that for the reaction with 500 nM wild type LipR.

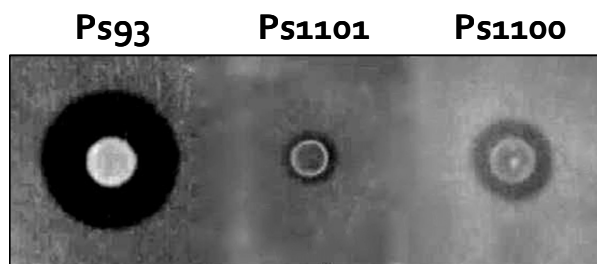


Figure 5. Effect of *rpoN* inactivation on lipase production. Lipase activity on a tributyrin oil plate for the Ps93 strain (wild type), the Ps1101 strain (Ps93 with inactivated *rpoN*), and the Ps1100 strain (Ps93 with inactivated *lipR*).

RpoN is involved in lipase production

To investigate the involvement of the RpoN protein in the regulation of lipase expression we created a *P. alcaligenes* mutant strain, Ps1101, with a disrupted *rpoN* gene. The *rpoN* gene was inactivated on the genomic DNA by insertional inactivation as described in experimental procedures. Southern hybridization analysis confirmed the presence of the integration insert into the chromosome (data not shown). One selected strain, named Ps1101, was used in further experiments. The effect of *rpoN* inactivation on lipolytic activity was studied with the indicator assay plates containing minimal medium agar with tributyrin (1% v/v). As shown in

Figure 5, the *rpoN*-inactivated strain, Ps1101, and the *lipR*-inactivated strain, Ps1100 displayed a remarkably reduced zone of clearance around the colonies in comparison to that seen for the Ps93 strain. The reduced clearance zone for the Ps1100 strain was already presented in our previous study¹⁶. The presence of the residual clearing zone for Ps1101 could be the result of low basal levels of lipase transcription or the result of resident esterase activity. This finding supports the hypothesis that lipase expression is governed by a σ^{54} -dependent transcription. Therefore, for optimal lipase expression both the RpoN factor and the LipR protein are required.

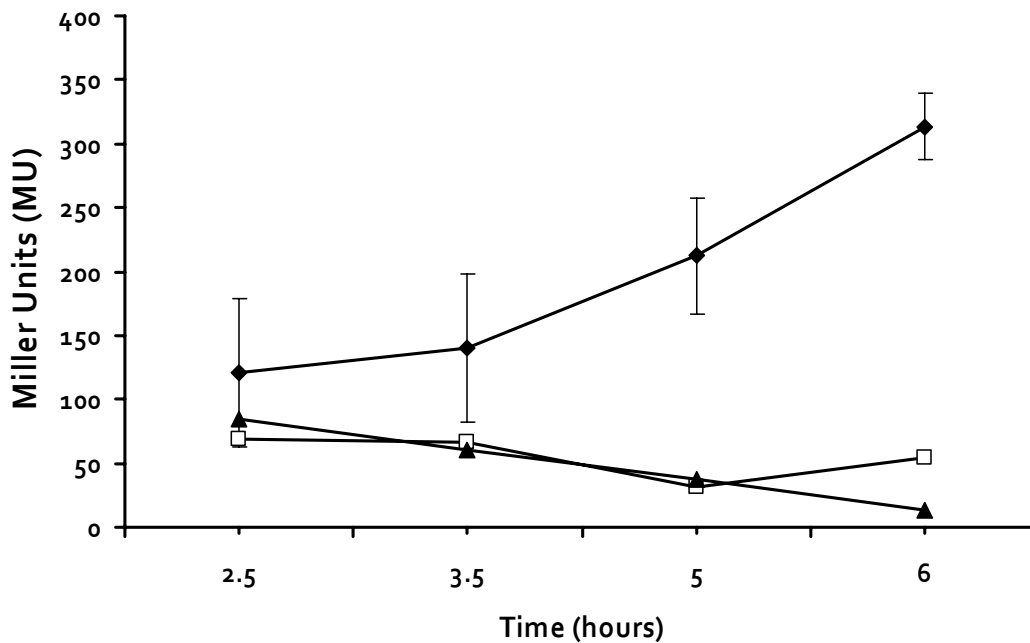


Figure 6. Analysis of the transcriptional activity of the *lipA* promoter (*lipA-lacZ*) in the *P. alcaligenes* wild type, *lipR* negative, and *rpoN* negative strains. The *lipA-lacZ* transcriptional fusion was used to determine the activation of the lipase promoter in cells of the parental (Ps93) and mutant strains (Ps1100, Ps1101) grown at 37°C in 2x TY medium. Samples were withdrawn at the times indicated (2.5 h, 3.5 h, 5 h, and 6 h). The strains used for the analysis were: Ps93 *lipA-lacZ* (closed diamonds), Ps1100 *lipA-lacZ* (open rectangles), and Ps1101 *lipA-lacZ* (closed triangles).

The transcription of *lipA* is dependent on LipR and RpoN

To assess whether the chromosomal inactivation of *lipR* and *rpoN* genes affected the lipase promoter activity, the pTZlipA vector bearing the *lipA-lacZ* transcriptional fusion was introduced to the *P. alcaligenes* strains (Ps93, Ps1100, and Ps1101) and β -galactosidase activity was measured after 2.5 h, 3.5 h, 5 h, and 6 h of growth. The levels of *lipA-lacZ* expression in the parental strain (Ps93) resulted in β -galactosidase activities higher than that observed for the Ps1100 and Ps1101 mutant strains (Figure 6). The *lipA-lacZ* expression levels in Ps1100 and

Ps1101 were comparable. Importantly, the inactivation of *lipR* and *rpoN* genes decreased the activation of lipase promoter and therefore showed that lipase expression is regulated by LipR and RpoN. This is in agreement with the observation of impaired lipase production on tributyrin plates for the Ps1100 mutant¹⁶ and the Ps1101 mutant (Figure 5) strains. The results presented here confirm that both LipR and RpoN are essential for transcription of lipase.

Discussion

Through this study, we investigated the activation of the *P. alcaligenes* lipase gene, *lipA*, by the LipR response regulator, a member of the NtrC-family of regulators. It was previously indicated that the UAS present upstream the -12/-24 promoter region functions as the LipR binding site and is therefore required for promoter activation¹⁶. Here, we demonstrate that LipR binds directly to the promoter region of lipase and that this binding is stimulated by LipR phosphorylation. Also, our data show that LipR displays the ATPase activity stimulated by phosphorylation and by the presence of DNA. Furthermore, the *rpoN* mutant strain displayed impaired lipase production as assayed by a tributyrin plate assay. Consistent with our previous studies¹⁶ and the latter observation, the analysis of the lipase promoter activation from the *lipA-lacZ* transcriptional fusion in the *P. alcaligenes* *lipR* negative and *rpoN* negative strains confirmed involvement of the two proteins, LipR and RpoN, in the lipase transcriptional activation.

Typically, bacterial response regulators, such as NtrC-like proteins, are activated by phosphorylation of an aspartate residue near their N-terminus by the histidine protein kinase. Phosphorylation stimulates an essential for transcriptional regulation oligomerization of the central domain. While structures of response regulators are known, little is understood about their activated phosphorylated forms, due to the intrinsic instability of the acid phosphate linkage^{2-4,35}. The lifetime (of seconds to hours) of phosphorylated response regulators is limited by a native autophosphatase activity of the response regulator and a phosphatase activity of the cognate kinase sensor. We haven't assessed the phosphorylation lifetime of LipR-P, yet we assume that the ratio of LipR-P to LipR will decrease over time due to the labile nature of aspartate phosphorylation.

The ATP requirement prior to initiation of transcription is not common for bacteria, except to activation of the σ^{54} -holoenzyme. The prokaryotic enhancer binding proteins, such as NtrC, are equipped with ATPase domains that catalyze open complex formation by σ^{54} -holoenzyme in an ATP-dependent manner^{20,36,37}. We anticipated that LipR exhibits an ATPase activity. To substantiate this hypothesis we performed an ATP hydrolysis assay with varying LipR and LipR-P concentrations and with addition of a DNA fragment (P_{lipA}199) containing the LipR binding site. Our data point out that LipR possesses an intrinsic ATPase activity, which is stimulated by phosphorylation and by the presence of DNA. In agreement with our results, it has been shown that phosphorylation of NtrC and the presence of DNA, containing specific NtrC binding sites, stimulated its ATPase activity^{37,38}. As well, the non-phosphorylated NtrC to some degree displayed ATPase activity³⁹. For many response regulators it has been shown that phosphorylation induces dimerization and/or cooperative binding to the respective target DNA sites^{37,40,41}. Accordingly, we examined the effect of *in vitro* LipR phosphorylation on its binding ability to the lipase promoter sequence. Our data from the gel retardation assay showed no

differences between LipR and LipR-P binding activities to the lipase promoter. Yet, our SPR experiment demonstrated that LipR poorly interacts with DNA when unphosphorylated and the LipR binding ability/affinity is stimulated by LipR phosphorylation. This is in accordance with results of others which show that phosphorylation of response regulators increases their binding ability to DNA⁴⁰⁻⁴². Perhaps, due to the relatively fast off-rate of LipR/LipR-P at the lipase promoter we were not able to detect any increase in binding between LipR and LipR-P to the lipase promoter in the gel retardation assay. It is plausible that the unstable LipR-DNA complexes dissociate prior to, or during, the running of the gel. This comparison shows that analyzing the binding of labile response regulators using SPR is superior.

Response regulators are normally phosphorylated by their cognate sensor kinases, yet many can also be phosphorylated *in vitro* at their conserved aspartate residue by low molecular weight phosphor donors, such as acetyl phosphate or carbamoyl phosphate⁴³⁻⁴⁵. This discovery facilitated the studies of response regulators without the requirement of their cognate kinases. It's noteworthy that in our studies next to phosphorylation of LipR by carbamoyl phosphate we also tested acetyl phosphate as a phosphor donor. Our results from the SPR experiment with LipR incubated with acetyl phosphate (data not shown), in contrast to carbamoyl phosphate, did not show any effect on LipR-DNA binding properties in comparison to untreated LipR. It might suggest that LipR displays specificity only for particular phosphor donors, such as carbamoyl phosphate. It has been previously demonstrated that response regulators may respond to various small molecule phosphor donors with different specificity^{43,46,47}.

Finally, the observation that LipR and RpoN are factors essential in the regulation of the expression of lipase from *P. alcaligenes* confirms our hypothesis and offers new possibilities to steer the lipase production levels at a higher scale. Yet, the present data do not exploit entirely the LipQR mode of action. Therefore, further studies should be aimed at clarifying the issues related to the LipR phosphorylation effect on its oligomerization state and DNA binding properties. Also, it would be important to define the exact stimuli of LipQ and its potential in LipR phosphorylation.

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References

1. Bourret, R.B., Borkovich, K.A., & Simon, M.I. Signal transduction pathways involving protein phosphorylation in prokaryotes. *Annu. Rev. Biochem.* 60, 401-441 (1991).
2. Parkinson, J.S. & Kofoid, E.C. Communication modules in bacterial signaling proteins. *Annu. Rev. Genet.* 26, 71-112 (1992).
3. Stock, J.B., Ninfa, A.J., & Stock, A.M. Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol. Rev.* 53, 450-490 (1989).
4. Parkinson, J.S. Signal transduction schemes of bacteria. *Cell* 73, 857-871 (1993).
5. Gerritse, G., Hommes, R.W.J., & Quax, W.J. Development of a lipase fermentation process that uses a recombinant *Pseudomonas alcaligenes* strain. *Appl. Environ. Microbiol.* 64, 2644-2651 (1998).

6. Jaeger, K.E. & Reetz, M.T. Microbial lipases form versatile tools for biotechnology. *Trends Biotechnol.* 16, 396-403 (1998).
7. Ghosh, P.K., Saxena, R.K., Gupta, R., Yadav, R.P., & Davidson, S. Microbial lipases: production and applications. *Sci. Prog.* 79 (Pt 2), 119-157 (1996).
8. Han, S.W., Nahas, E., & Rossi, A. Regulation of synthesis and secretion of acid and alkaline phosphatases in *Neurospora crassa*. *Curr. Genet.* 11, 521-527 (1987).
9. Oso, B.A. Utilization of lipids as sole carbon sources by thermophilic fungi. *Z. Allg. Mikrobiol.* 14, 713-717 (1974).
10. Tanaka, J., Sudo, T., Ihara, F., Nihira, T., & Yamada, Y. Increase production of lactonizing lipase (LipL) from *Pseudomonas* sp. strain 109 by lipids and detergents. *Biosci. Biotechnol. Biochem.* 63, 900-904 (1999).
11. Gerritse, G., Ure, R., Bizoullier, F., & Quax, W.J. The phenotype enhancement method identifies the Xcp outer membrane secretion machinery from *Pseudomonas alcaligenes* as a bottleneck for lipase production. *J. Biotechnol.* 64, 23-38 (1998).
12. Chihara-Siomi, M., Yoshikawa, K., Oshima-Hirayama, N., Yamamoto, K., Sogabe, Y., Nakatani, T., Nishioka, T., & Oda, J. Purification, molecular cloning, and expression of lipase from *Pseudomonas aeruginosa*. *Arch. Biochem. Biophys.* 296, 505-513 (1992).
13. Frenken, L.G., Bos, J.W., Visser, C., Muller, W., Tommassen, J., & Verrips, C.T. An accessory gene, *lipB*, required for the production of active *Pseudomonas glumae* lipase. *Mol. Microbiol.* 9, 579-589 (1993).
14. Beven, C.A., Dieckelmann, M., & Beacham, I.R. A strain of *Pseudomonas fluorescens* with two lipase-encoding genes, one of which possibly encodes cytoplasmic lipolytic activity. *J. Appl. Microbiol.* 90, 979-987 (2001).
15. Cox, M., Gerritse, G., Dankmeyer, L., & Quax, W.J. Characterization of the promoter and upstream activating sequence from the *Pseudomonas alcaligenes* lipase gene. *J. Biotechnol.* 86, 9-17 (2001).
16. Krzeslak, J., Gerritse, G., van Merkerk, R., Cool, R.H., & Quax, W.J. Lipase expression in *Pseudomonas alcaligenes* is under the control of a two-component regulatory system. *Appl. Environ. Microbiol.* 74, 1402-1411 (2008).
17. Lenz, O., Strack, A., Tran-Betcke, A., & Friedrich, B. A hydrogen-sensing system in transcriptional regulation of hydrogenase gene expression in *Alcaligenes* species. *J. Bacteriol.* 179, 1655-1663 (1997).
18. Genschik, P., Drabikowski, K., & Filipowicz, W. Characterization of the *Escherichia coli* RNA 3'-terminal phosphate cyclase and its sigma₅₄-regulated operon. *J. Biol. Chem.* 273, 25516-25526 (1998).
19. Weiner, L., Brissette, J.L., & Model, P. Stress-induced expression of the *Escherichia coli* phage shock protein operon is dependent on sigma₅₄ and modulated by positive and negative feedback mechanisms. *Genes Dev.* 5, 1912-1923 (1991).
20. Kustu, S., Santero, E., Keener, J., Popham, D., & Weiss, D. Expression of sigma-54 (NtrA) dependent genes is probably united by a common mechanism. *Microbiol. Rev.* 53, 367-376 (1989).
21. Chen, B., Doucleff, M., Wemmer, D.E., De Carlo, S., Huang, H.H., Nogales, E., Hoover, T.R., Kondrashkina, E., Guo, L., & Nixon, B.T. ATP ground- and transition states of bacterial enhancer binding AAA+ ATPases support complex formation with their target protein, sigma₅₄. *Structure* 15, 429-440 (2007).
22. Lee, S.Y., De La, T.A., Yan, D., Kustu, S., Nixon, B.T., & Wemmer, D.E. Regulation of the transcriptional activator NtrC1: structural studies of the regulatory and AAA+ ATPase domains. *Genes Dev.* 17, 2552-2563 (2003).
23. Kern, D., Volkman, B.F., Luginbuhl, P., Nohaile, M.J., Kustu, S., & Wemmer, D.E. Structure of a transiently phosphorylated switch in bacterial signal transduction. *Nature* 402, 894-898 (1999).
24. Meyer, M.G., Park, S., Zeringue, L., Staley, M., McKinstry, M., Kaufman, R.I., Zhang, H., Yan, D., Yennawar, N., Yennawar, H., Farber, G.K., & Nixon, B.T. A dimeric two-component receiver domain inhibits the sigma₅₄-dependent ATPase in DctD. *FASEB J.* 15, 1326-1328 (2001).
25. Park, S., Meyer, M., Jones, A.D., Yennawar, H.P., Yennawar, N.H., & Nixon, B.T. Two-component signaling in the AAA + ATPase DctD: binding Mg²⁺ and BeF₃⁻ selects between alternate dimeric states of the receiver domain. *FASEB J.* 16, 1964-1966 (2002).
26. Sambrook, J.F.E.F.a.M.T. Molecular cloning: a laboratory manual. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; 1989).
27. Wirth, R., Friesenegger, A., & Fiedler, S. Transformation of various species of gram-negative bacteria belonging to 11 different genera by electroporation. *Molecular & General Genetics* 216, 175-177 (1989).

28. Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., & Boyer, H.W. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* 2, 95-113 (1977).
29. Schweizer, H.P. & Chuanchuen, R. Small broad-host-range lacZ operon fusion vector with low background activity. *Biotechniques* 31, 1258, 1260, 1262 (2001).
30. Heeb, S., Blumer, C., & Haas, D. Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J. Bacteriol.* 184, 1046-1056 (2002).
31. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254 (1976).
32. Ebbole, D.J. & Zalkin, H. Interaction of a putative repressor protein with an extended control region of the *Bacillus-subtilis* pur operon. *J. Biol. Chem.* 264, 3553-3561 (1989).
33. Parlato, G., Guesnet, J., Crechet, J.B., & Parmeggiani, A. The GTPase activity of elongation factor Tu and the 3'-terminal end of aminoacyl-tRNA. *FEBS Lett.* 125, 257-260 (1981).
34. Miller, J.H. Experiments in molecular genetics. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; 1972).
35. Keener, J. & Kustu, S. Protein kinase and phosphoprotein phosphatase activities of nitrogen regulatory proteins NTRB and NTRC of enteric bacteria: roles of the conserved amino-terminal domain of NTRC. *Proc. Natl. Acad. Sci. U. S. A* 85, 4976-4980 (1988).
36. Popham, D.L., Szeto, D., Keener, J., & Kustu, S. Function of a bacterial activator protein that binds to transcriptional enhancers. *Science* 243, 629-635 (1989).
37. Weiss, D.S., Batut, J., Klose, K.E., Keener, J., & Kustu, S. The phosphorylated form of the enhancer-binding protein NTRC has an ATPase activity that is essential for activation of transcription. *Cell* 67, 155-167 (1991).
38. Austin, S. & Dixon, R. The prokaryotic enhancer binding protein NTRC has an ATPase activity which is phosphorylation and DNA dependent. *EMBO J.* 11, 2219-2228 (1992).
39. Kustu, S., North, A.K., & Weiss, D.S. Prokaryotic transcriptional enhancers and enhancer-binding proteins. *Trends Biochem. Sci.* 16, 397-402 (1991).
40. Fiedler, U. & Weiss, V. A common switch in activation of the response regulators NtrC and PhoB: phosphorylation induces dimerization of the receiver modules. *EMBO J.* 14, 3696-3705 (1995).
41. Huang, K.J., Lan, C.Y., & Igo, M.M. Phosphorylation stimulates the cooperative DNA-binding properties of the transcription factor OmpR. *Proc. Natl. Acad. Sci. U. S. A* 94, 2828-2832 (1997).
42. Aiba, H., Nakasai, F., Mizushima, S., & Mizuno, T. Phosphorylation of a bacterial activator protein, OmpR, by a protein kinase, EnvZ, results in stimulation of its DNA-binding ability. *J. Biochem.* 106, 5-7 (1989).
43. Lukat, G.S., McCleary, W.R., Stock, A.M., & Stock, J.B. Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors. *Proc. Natl. Acad. Sci. U. S. A* 89, 718-722 (1992).
44. McCleary, W.R. & Stock, J.B. Acetyl phosphate and the activation of two-component response regulators. *J. Biol. Chem.* 269, 31567-31572 (1994).
45. Deretic, V., Leveau, J.H., Mohr, C.D., & Hibler, N.S. *In vitro* phosphorylation of AlgR, a regulator of mucoidy in *Pseudomonas aeruginosa*, by a histidine protein kinase and effects of small phospho-donor molecules. *Mol. Microbiol.* 6, 2761-2767 (1992).
46. Molle, V. & Buttner, M.J. Different alleles of the response regulator gene bldM arrest *Streptomyces coelicolor* development at distinct stages. *Mol. Microbiol.* 36, 1265-1278 (2000).
47. Schar, J., Sickmann, A., & Beier, D. Phosphorylation-independent activity of atypical response regulators of *Helicobacter pylori*. *J. Bacteriol.* 187, 3100-3109 (2005).