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Physiological Factors Affecting Production of Extracellular Lipase (LipA) in *Acinetobacter calcoaceticus* BD413: Fatty Acid Repression of *lipA* Expression and Degradation of LipA

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The extracellular lipase (LipA) produced by *Acinetobacter calcoaceticus* BD413 is required for growth of the organism on triolein, since mutant strains that lack an active lipase fail to grow with triolein as the sole carbon source. Surprisingly, extracellular lipase activity and expression of the structural lipase gene (*lipA*), the latter measured through *lacZ* as a transcriptional reporter, are extremely low in triolein cultures of LipA$^+$ strains. The explanation for this interesting paradox lies in the effect of fatty acids on the expression of *lipA*. We found that long-chain fatty acids, especially, strongly repress the expression of *lipA*, thereby negatively influencing the production of lipase. We propose the involvement of a fatty acyl-responsive DNA-binding protein in regulation of expression of the *A. calcoaceticus lipBA* operon. The potential biological significance of the observed physiological competition between expression and repression of *lipA* in the triolein medium is discussed. Activity of the extracellular lipase is also negatively affected by proteolytic degradation, as shown in in vitro stability experiments and by Western blotting (immunoblotting) of concentrated supernatants of stationary-phase cultures. In fact, the relatively high levels of extracellular lipase produced in the early stationary phase in media which contain hexadecane are due only to enhanced stability of the extracellular enzyme under those conditions. The rapid extracellular degradation of LipA of *A. calcoaceticus* BD413 by an endogenous protease is remarkable and suggests that proteolytic degradation of the enzyme is another important factor in regulating the level of active extracellular lipase.

The gram-negative soil bacterium *Acinetobacter calcoaceticus* BD413 produces a single extracellular lipase (EC 3.1.1.3) along with several other (cell-bound) lipolytic enzymes (22–24). The extracellular lipase (LipA) has recently been purified and characterized, and the encoding *lipA* gene has been cloned (23). Secretion of LipA requires the activity of LipB, a specific LipA chaperone that is anchored into the cytoplasmic membrane and faces the periplasm (5, 24). Both the lipase and the lipase chaperone of *A. calcoaceticus* have high degrees of sequence identity with their respective counterparts in pseudomonads, suggesting strong similarities in the lipase production mechanisms in these genera. Indeed, identification of several similar components involved in the secretion of lipase has allowed us to characterize the mechanism of lipase production by *A. calcoaceticus* in considerable detail (23, 24).

Despite the striking resemblance of *Acinetobacter* and several *Pseudomonas* species with regard to their mechanisms of lipase production, an important difference has been unveiled by the recent discovery that the structural lipase gene in *A. calcoaceticus* BD413 is located downstream of the chaperone gene (*lipBA* [24]). Compared with *Pseudomonas* species, the chromosomal order of the two genes is reversed in *Acinetobacter* (4, 13–15, 19, 24). This difference in gene order has important consequences for the regulation of their expression, since in both organisms the two genes are located in a bicistronic operon (4, 24). The promoter of the *A. calcoaceticus lipBA* operon, identified via primer extension analysis, was shown to be similar to the recognition site for the *Escherichia coli* vegetative sigma factor (σ^A [24]). This finding suggests σ^A-type control of *lipBA* expression in *A. calcoaceticus* BD413.

The lipase chaperone is thought to mediate formation of a low level of secondary structure in the LipA polypeptide and to protect the immature LipA against proteolytic degradation (5). In addition, LipB presumably prevents complete folding of the LipA polypeptide before it has crossed the outer membrane (24). In doing so, LipB prevents formation of active lipase prior to complete secretion. This is most likely required for safety reasons, since the functional lipase could easily devastate membranes. The chaperone is therefore not only required for production of lipase but also essential to the lipase-producing cell. Despite such intricate safeguards during LipA secretion, production of lipase remains a risky trait, even after full extrusion of the hydrolytic enzyme. It is therefore reasonable to expect additional mechanisms of regulation of lipase production, for instance, at the level of *lipBA* expression. In addition, specific physiological conditions may support the formation of lipase. Thus, *A. calcoaceticus* BD413 forms particularly high levels of extracellular lipase when grown in a medium containing long-chain alkanes such as hexadecane (23).

The effects of a wide variety of environmental conditions on the production of lipases of both fungal and bacterial origin have been investigated (for reviews, see references 18 and 33). However, such studies have thus far mainly aimed at optimization of lipase production, rather than providing a general understanding of the genetic and physiological mechanisms affecting extracellular lipase formation. Similarly, though lipase-

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TABLE 1. Strains of *E. coli* and *A. calcoaceticus* and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics[a]	Marker(s)	Reference
E. coli DH5α	Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *recA1 thi-1 relA1*		9
A. calcoaceticus			
BD413	Wild type		20
AAC320-1	*lipA*::(4.7-kbp pAVP21 cassette); *lipBA*::*lacZ lipA*	Km^r	This paper
AAC321-1	BD413::pALJA436-1 (single crossover at *lipA*); *lipBA*::*lacZ*; $LipA^+$	Km^r	This paper
Plasmids			
pALJA432	pUN121, *Eco*RV/*Sal*I::*lipBA* (*Eco*RV/*Sal*I)	Amp^r	23
pALJA434	pALJA432, *lipA*-*Eco*RI	Amp^r	This paper
pALJA436-1	pALJA434, *Eco*RI-blunt::(4.7-kbp pAVP21 cassette, *Sal*I-blunt), *lipA*::*lacZ*	Amp^r Km^r	This paper
pAVP21	4.7-kbp promoterless *lacZ-nptII* (=pAVP21 cassette)	Amp^r Km^r	36

[a] For construction of strains and plasmids, see Materials and Methods. *nptII*, the kanamycin resistance gene of pAVP21; Amp^r and Km^r resistance to ampicillin and kanamycin, respectively.

encoding genes from a wide variety of organisms have been cloned and analyzed (18), few studies with regard to the regulation of their expression have been published. This is remarkable in view of the generally accepted notion that prokaryotic processes are primarily regulated at the level of expression of the genes involved.

We have compared the levels of expression of *lipBA* and the formation of extracellular lipase in response to different carbon sources. Chromosomal expression of *lipBA* was measured via generation of transcriptional *lipBA*::*lacZ* fusion strains. *A. calcoaceticus* BD413, unlike most *Pseudomonas* strains, is highly suitable for such studies because of its natural transformability (20, 29). Expression of *lipBA* appears to be strongly regulated. In addition, evidence is mounting that instability of the extracellular LipA protein is an important factor determining the level of active extracellular lipase. The nature of both regulatory mechanisms supports the notion that lipase production is a delicate process that requires many levels of control.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used are listed in Table 1.

Media and culture conditions. Luria-Bertani medium (LB; prepared as described previously [22]) was routinely used as the growth and transformation medium for strains of *A. calcoaceticus* and for growth of *E. coli* strains. Antibiotics were used in the following final concentrations in LB plates (1.5% [wt/vol] agar) and in liquid LB medium: ampicillin, 100 μg/ml; and kanamycin, 15 μg/ml for *A. calcoaceticus* and 50 μg/ml for *E. coli*. Nutrient broth (NB; Gibco) plates with 1% (wt/vol) NaCl and 1% (vol/vol) emulsified egg yolk (Oxoid) were used for detection of lipase (LipA) production by colonies of *A. calcoaceticus* as described previously (22, 23).

For determination of lipase- and β-galactosidase production in batch cultures, *A. calcoaceticus* was grown in mineral medium. This medium was essentially prepared as described by Juni (21) and contained (per liter) 11 mM KH_2PO_4, 95 mM Na_2HPO_4, 0.81 mM $MgSO_4$, 37 mM NH_4Cl, 68 μM $CaCl_2$, and 1.8 μM $FeSO_4$. Specific carbon sources, as lactic acid (either 110 or 28 mM), triolein (0.5% [vol/vol]), or oleic acid (0.2 to 10 mM), were added to this medium prior to autoclaving. The pH was adjusted to 7.0, and the medium was autoclaved at 120°C for 20 min. When hexadecane (34 mM, final concentration) was used as the sole carbon and energy source, it was added as a sterile emulsion (containing 680 mM hexadecane and 10% [wt/vol] gum arabic) to the separately autoclaved medium. This hexadecane-gum arabic mixture was emulsified in 50-ml portions with a Branson 250 Sonifier equipped with a microtip for 3 min at 75 W (duty cycle, 100%) and prior to autoclaving at 120°C for 20 min. The effect of short- and medium-chain fatty acids on expression of the *lipA* gene (see Fig. 6) was measured in cultures with 110 mM lactic acid and 3 mM butyric acid (C_4), caprylic acid (C_6), caprolic acid (C_8), or lauric acid (C_{12}).

Strains of *A. calcoaceticus* were grown either in Erlenmeyer flasks on a gyratory shaker or in MultiGen Fermenter vessels (New Brunswick Scientific), with saturating aeration at 30°C. To prevent lag-phase problems, cultures to be used for measuring growth, lipase production, and/or production of β-galactosidase were inoculated from cultures which had been adapted to the same medium through two previous batch-growth periods. When appropriate, growth in these cultures was measured spectrophotometrically at 580 nm, either directly on diluted culture samples or (in the case of samples from triolein or hexadecane cultures) after cells were washed twice in a buffer containing 50 mM Tris (adjusted to pH 7.5 with HCl) and 2 mM $MgCl_2$ and resuspended in a fixed volume of the same buffer.

Chemicals. All chemicals were of highest quality commercially available. Restriction enzymes and T4 DNA ligase were obtained from Pharmacia Biotech, as were custom-synthesized primers used for site-directed mutagenesis and sequence determination.

Recombinant DNA techniques. All recombinant DNA techniques, including isolation of chromosomal and plasmid DNA and introduction of plasmid DNA into *E. coli* or *A. calcoaceticus* via transformation, were essentially performed as described previously (22–24, 31).

Construction of *lipA*::*lacZ* fusion plasmid pALJA436-1 and subsequent generation of *lipA*::*lacZ* fusion strains AAC320-1 and AAC321-1. The site-directed mutagenesis strategy described previously for the generation of the LipA Ser-99-to-Ala mutation in strain AAC316 (23), based on the megaprimer method described by Landt et al. (26), was applied to generate an *Eco*RI restriction site just downstream of the translation initiation codon of the *lipA* open reading frame (ORF) in plasmid pALJA432 (Table 1). This site could then be used for introduction of a selectable marker, to disrupt the lipase gene, and to construct a transcriptional *lipA*::*lacZ* fusion. All reaction conditions were as described earlier (23). In this case, primer lip24Eco (5′-GTAAATAATAATTTGAATTC CATTTTATTTCCC-3′; the changed base is doubly underlined) was used as the mutagenesis primer. This primer introduces a G as the fourth base in the *lipA* ORF (resulting in a change in the second codon, from AAA to GAA) and generates an *Eco*RI site (underlined in the primer sequence) after the third base in *lipA*. Primers lip24Eco and 5′ primer lip23 (5′-GCACCACAGGTACAGAT TCGGG-3′), which anneals 178 bases upstream of *lipA*, were used in the first series of thermocycle reactions to generate the 231-bp megaprimer lip2a. Megaprimer lip2a was isolated as described previously for isolation of lip1a (23) and was used in the second series of thermocycle reactions together with 3′ primer lip25 (5′-CATGAAGGTACCAGCCCGTC-3′ [23]). The resulting 1,023-bp fragment, lip2b, was gel purified and contained the *Eco*RI site generated by primer lip24Eco. Subsequently, fragment lip2b was digested with *Hin*dIII and *Kpn*I, and the restriction fragment was ligated into *Hin*dIII-*Kpn*I-digested pALJA432, yielding plasmid pALJA434 (Fig. 1). The ligation mixture was transformed into *E. coli* DH5α, with selection on plates containing ampicillin. The sequence upstream of the *Eco*RI restriction site introduced in pALJA434 was unchanged, as verified by sequence analysis.

To generate a transcriptional *lipA*::*lacZ* fusion, the *Eco*RI site generated in pALJA434 was used for insertion of a cassette of pAVP21 (36), carrying a promoterless *lacZ* gene and the *nptII* gene encoding resistance to kanamycin (*lacZ*/Km cassette). This cassette (isolated as a 4.7-kbp *Sal*I fragment, with sticky ends filled in with the Klenow fragment of DNA polymerase I) was ligated into the unique *Eco*RI site of pALJA434 (sticky ends were again filled in with the Klenow fragment). *E. coli* DH5α transformants forming blue colonies on plates containing ampicillin, kanamycin, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were identified as carrying the intended *lipA*::*lacZ* fusion plasmid pALJA436-1 (Fig. 1). Transfer of the *lacZ*/Km insertion of pALJA436-1 to the *Acinetobacter* chromosome was achieved via linearization of pALJA436-1 with *Sca*I (cleaves the vector part of pALJA436-1) and subsequent introduction of the linearized plasmid into *A. calcoaceticus* BD413 via natural transformation. Selection for strains carrying the *lipA*::*lacZ*/Km construct inserted into the chromosome, via homologous recombination at flanking chromosomal sequences, was performed on NB egg yolk plates (to screen for lipase activity; see above) containing kanamycin. One such strain, forming blue colonies on the selection plates, was designated AAC320-1 (Fig. 2A). This strain did not form turbid zones around colonies on egg yolk plates, showing that insertion of the *lacZ*/Km cassette had occurred behind the translation initiation codon of the *lipA* gene, thereby disrupting the *lipA* ORF and thus generating a lipase-deficient mutant.

To generate an *Acinetobacter* strain that carried the *lacZ*/Km insertion into *lipA*, in addition to a wild-type copy of the lipase-encoding gene, plasmid

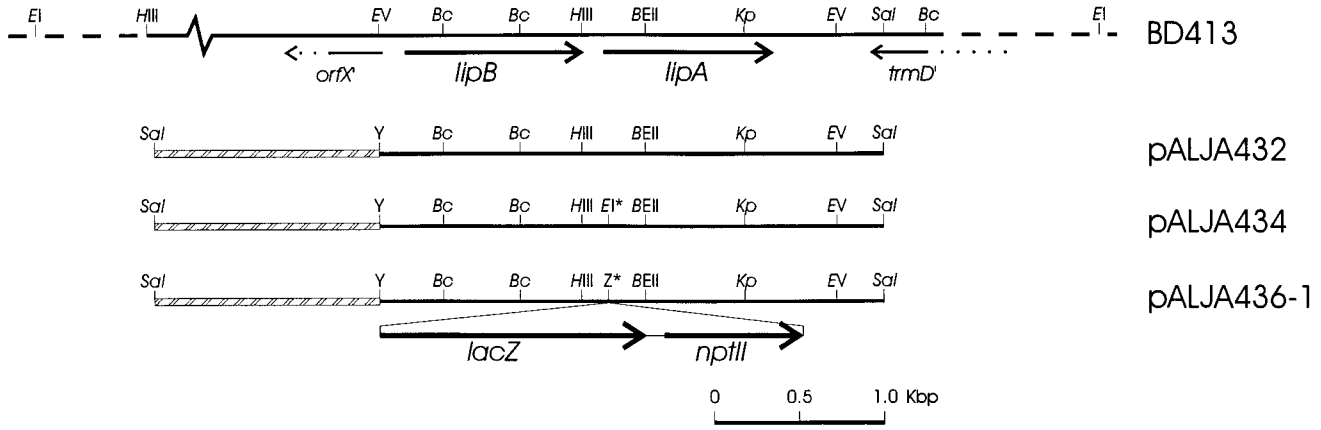

FIG. 1. Schematic representation of the lipase-encoding region on the chromosome of *A. calcoaceticus* BD413 and on different plasmids. Black thin bars represent *A. calcoaceticus* DNA; hatched boxes represent fragments derived from vector pUN121. Restriction sites: *Bc*, *Bcl*I; *B*EII, *Bst*EII; *E*I, *Eco*RI; *E*V, *Eco*RV; *H*III, *Hin*dIII; *Kp*, *Kpn*I; *Sal*, *Sal*I; Y, *Eco*RV-*Sma*I ligation site; *EI**, *Eco*RI site introduced just downstream of the *lipA* translation initiation site; *Z**, ligation site between *Eco*RI (blunt ends generated with Klenow fragment) and *Sal*I (blunt ends generated). ORFs are indicated by arrows; *lipB* is the gene encoding the specific lipase chaperone (24). The promoterless *lacZ* gene and the *nptII* gene encoding kanamycin resistance refer to the cassette of pAVP21, inserted just downstream of the *lipA* translation initiation codon in pALJA434, creating a transcriptional *lipA*::*lacZ* fusion. Only the chromosomal fragments between the left-hand *Eco*RV site and the right-hand *Bcl*I site have been drawn to scale. For further details, see text.

pALJA436-1 was transformed into *A. calcoaceticus* BD413 directly, without prior linearization of the plasmid. Selection for integration of intact pALJA436-1 into the chromosome of BD413, by a single crossover recombination event (Fig. 2B), took place on egg yolk plates with kanamycin and X-Gal. From these plates, we selected several blue colonies that formed a turbid zone, indicative of LipA activity. One of the strains showing this phenotype, in which pALJA436-1 had integrated at *lipA* sequences, was designated AAC321-1. In AAC321-1, the *lipB-lipA*::*lacZ* fusion cassette is located downstream of the wild-type *lipBA* operon on the chromosome (Fig. 2B). The exact genotypes of AAC320-1 and AAC321-1, as shown in Fig. 2, were verified via Southern hybridization of digested chromosomal DNA of the two mutant strains, using the *lacZ*/Km cassette or fragments of the *lipA* region as a probe (not shown).

Measurement of extracellular lipase activity and intracellular β-galactosidase activity. Lipase activity was determined in culture supernatants as described previously (22, 23), using *p*-nitrophenyl palmitate as the substrate. One unit of lipase activity is defined as the amount of enzyme forming 1 μmol of *p*-nitrophenol per minute. Volumetric lipase production was calculated as units per liter of culture and divided by the optical density at 580 nm (OD_{580}) of the culture sample, in order to obtain the specific lipase activity (U/LOD). Production of β-galactosidase activity was recorded as a measure for expression of the *lipA* gene in cultures of *A. calcoaceticus lipA*::*lacZ* fusion strains AAC320-1 and AAC321-1. Cells were harvested from the medium via centrifugation, washed once (regular procedure) or twice (only for samples from triolein or hexadecane cultures; see above) in a buffer containing 50 mM Tris HCl (pH 7.5) and 2 mM $MgCl_2$, and resuspended in a known volume of the same buffer, for which the OD_{580} value was recorded, and the samples were frozen until further use. Determination of specific β-galactosidase activity (given in Miller units) was performed essentially as described by (27), using *o*-nitrophenyl-β-D-galactopyranoside as the substrate. All reactions were performed with duplicate samples from two independent cultures. The values of specific lipase and β-galactosidase activity shown in Fig. 3 to 5 are derived from samples of single cultures, of which the pattern of production of enzyme activity does not deviate significantly from that of the duplicate culture.

SDS-PAGE and immunodetection of LipA on Western blots (immunoblots). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed essentially by the method of Laemmli (25) as described previously (23). For immunodetection of LipA, proteins from SDS-polyacrylamide gels were blotted onto nitrocellulose by the method of Towbin et al. (35) as described previously (23). For detection of the extracellular lipase in cultures grown in NB medium, cells were separated from the medium via centrifugation and proteins in the supernatant were concentrated via $(NH_4)_2SO_4$ precipitation: to culture supernatant, solid $(NH_4)_2SO_4$ was added to 60% (wt/vol) saturation while stirring gently at 4°C. This suspension was then centrifuged at 10,000 × *g* (10 min, 4°C), and the pellet containing the lipase was dissolved in the smallest possible volume of 10 mM Tris (HCl, pH 8.0) and dialyzed against the same buffer (at least 2 h at 4°C). The lipase activity in supernatants was thus concentrated approximately 50-fold. Western blots were immunostained by using polyclonal rabbit antibodies, raised against the lipase (23), and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G, in combination with horseradish peroxidase color development reagent as recommended by the supplier (Bio-Rad). Serum with the primary antibody was diluted 1,000- to 2,000-fold before use.

RESULTS

Generation of *lipA*::*lacZ* fusion strains of *A. calcoaceticus* BD413. To be able to specifically study the expression of *lipA* of *A. calcoaceticus* BD413, we generated strains in which a promoterless *lacZ* gene was inserted in the *lipA* ORF on the *Acinetobacter* chromosome. This places *lacZ* under transcriptional control of the *lipBA* promoter (see Materials and Methods for details). Strain AAC320-1 carries this *lipA*::*lacZ* fusion on the chromosome and no longer produces lipase because of the disruption of *lipA*. Since LipA activity is absolutely required for growth on carbon sources such as triolein (see below), expression of *lipA* during growth on this medium cannot be measured via a classic single reporter gene fusion such as present in AAC320-1. Therefore, we also constructed a second *lipA*::*lacZ* fusion strain (AAC321-1) which carries both the *lipA*::*lacZ* fusion and a functional *lipBA* operon (Fig. 2). The latter strain still produces lipase as does wild-type BD413. Together with BD413, strains AAC320-1 and AAC321-1 were used to simultaneously investigate physiological factors that control the production of lipase in *A. calcoaceticus* at the level of expression of the *lipA* gene (AAC320-1 and AAC321-1) and extracellular LipA activity (BD413 and AAC321-1). Though measurements of β-galactosidase activity in strains AAC320-1 and AAC321-1 reflect the expression of the entire *lipBA* operon of *A. calcoaceticus* BD413 (24), it will be referred to as *lipA* expression throughout this report.

Triolein, a growth substrate that requires LipA for degradation, does not support high lipase production. Lipase activity produced by *A. calcoaceticus* BD413 can be detected on agar plates containing different lipidic substrates (22, 24). Although LipA has a strong preference for cleavage of long-chain ester derivatives of *p*-nitrophenol in vitro (23), it was noted that short-chain triglycerides such as tributyrin served as better substrates for in vivo detection of lipase activity on plates than did long-chain triglycerides such as triolein. While tributyrin can also be degraded by several of the esterases produced by *A.*

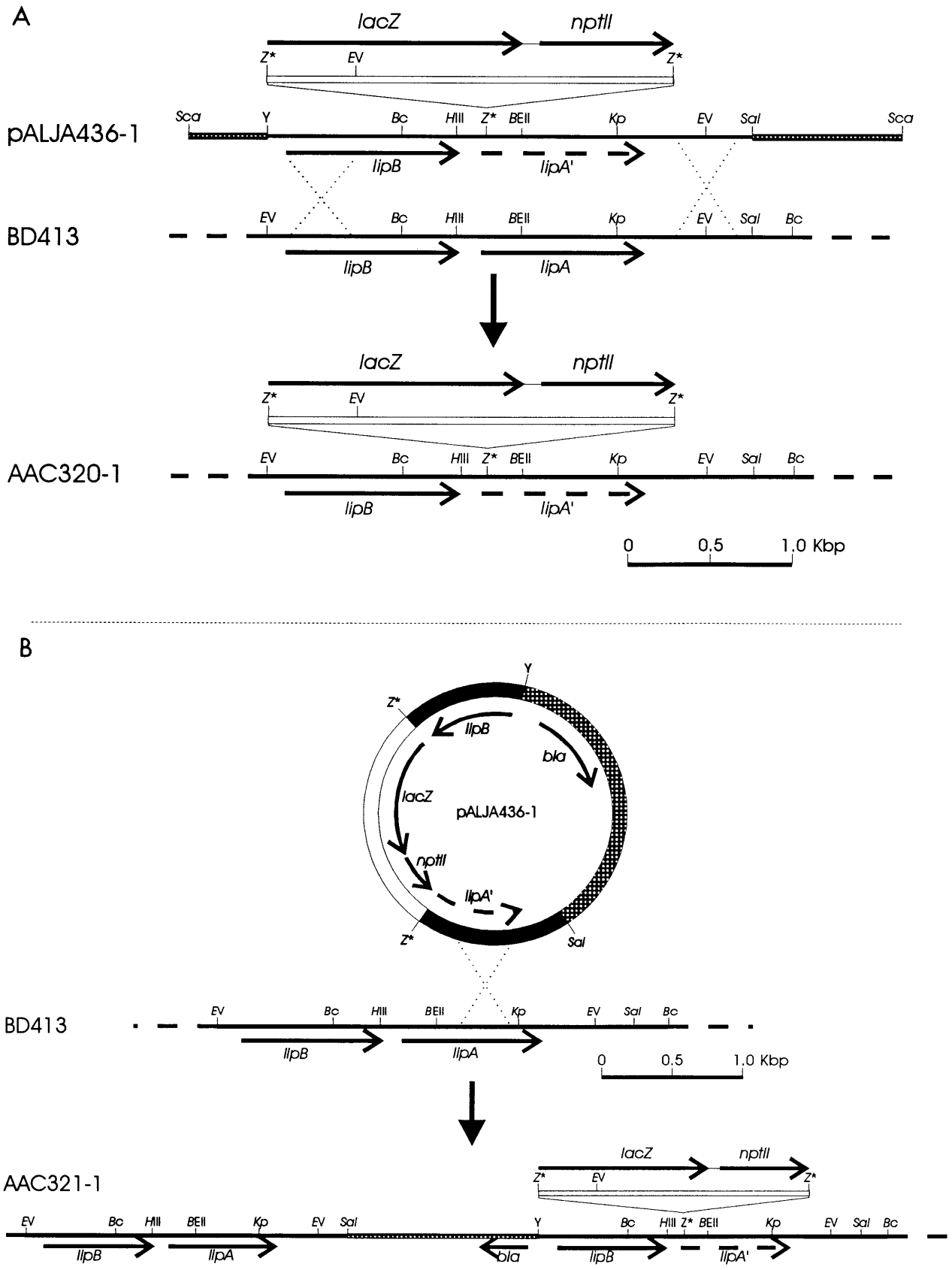

FIG. 2. Schematic representation of the recombination events that occurred during construction of *A. calcoaceticus lipA*::*lacZ* fusion strains AAC320-1 (A) and AAC321-1 (B) with plasmid pALJA436-1 (Fig. 1). (A) Via a double crossover event, *lipA* is replaced by the *lipA*::*lacZ* fusion cassette of linearized pALJA436-1. As a consequence, AAC320-1 does not possess a functional *lipA* gene (represented by a broken arrow) and is therefore lipase negative. (B) In AAC321-1, a single crossover event has occurred, during which the intact pALJA436-1 is inserted into the chromosome, such that a functional *lipBA* unit is retained, next to the *lipA*::*lacZ* fusion cassette of pALJA436-1. Consequently, AAC321-1 expresses a functional *lipA* and is lipase positive. For abbreviations, see the legend to Fig. 1.

calcoaceticus BD413 (22, 24), thus far LipA has been the only enzyme with detectable activity toward triolein. BD413 can use this triglyceride as the sole carbon and energy source in mineral media. To investigate whether the lipase was in fact required for growth on triolein, we checked growth of *lipA*-negative strain AAC320-1 in mineral medium with triolein as the sole carbon source. No growth could be detected in this triolein medium (OD_{580} after 12 h = 0.02). Likewise, lipase-negative strain *A. calcoaceticus* AAC316 failed to grow on triolein (not shown). The latter strain produces an inactive lipase protein that differs from the wild-type enzyme in that the active-site serine has been changed to an alanine through site-specific mutagenesis (23). A similar result was obtained in cultures with olive oil (a crude substitute for triolein) as the carbon source, although in this case a slight increase in biomass was observed with both strains (data not shown), indicating that some growth occurred on contaminating substrates in the olive oil. In contrast with the lipase-negative mutants, lipase-positive strains BD413 and AAC321-1 both reached high biomass levels in triolein (Fig. 3A; OD_{580} after 11 h = 7.8) and olive oil (not shown). These results indicate that LipA is essential for growth on the long-chain triglyceride triolein.

Despite the requirement of LipA activity for degradation of triolein to occur, surprisingly, only very low levels of lipase activity could be detected during growth in the triolein cultures of BD413 and AAC321-1 (Fig. 3B). Specific lipase activity slowly decreases to as low as 30 U/LOD in the late exponential phase. Similarly low levels of lipase activity were found during growth on olive oil (not shown). This is in sharp contrast with the high levels of extracellular lipase found in a medium with for instance the nonlipase substrate hexadecane as the carbon and energy source (up to 2,000 U/LOD [Fig. 4]).

The low lipase level during growth of *A. calcoaceticus* on triolein can be explained by a relatively low level of expression of the *lipA* gene, as relatively little β-galactosidase activity (specific activity of around 100 Miller units) could be detected during logarithmic growth of strain AAC321-1 on the triglyceride (Fig. 3C). This low-level expression of *lipA* is followed by a slight increase at the onset of the stationary phase (Fig. 3C), which is perfectly reflected in an increase in specific extracellular lipase activity (Fig. 3B). The observed low levels of *lipA* expression and extracellular LipA activity are remarkable since LipA activity is required for growth on triolein. These observations suggest that compounds liberated during growth on triolein are strong inhibitors of lipase production at the level of *lipA* transcription.

Lipase production and *lipA* expression during growth on hexadecane. Previously, *A. calcoaceticus* BD413 has been shown to produce high amounts of extracellular lipase when grown in a mineral medium with the nonlipase substrate hexadecane as the sole carbon and energy source (23). To compare the levels of lipase production and *lipA* expression during growth on hexadecane, strains BD413, AAC320-1, and AAC321-1 were grown in batch cultures in the mineral hexadecane medium. Figure 4A shows that all three strains grow rapidly with hexadecane as the sole carbon and energy source and reach comparable cell densities. This finding confirms earlier results indicating that the lipase is not required for growth on hexadecane (24). The appearance of extracellular lipase activity in this medium follows a growth-phase-dependent pattern in cultures of both BD413 and AAC321-1 (Fig. 4B). Lipase levels are comparably low (around 400 U/LOD) at t = 13.5 h, presumably near the onset of the stationary phase, in cultures of both lipase-producing strains. Further incubation during the stationary phase leads to a strong induction of lipase production, though to variable levels (between 1,500 and 2,000 U/LOD for BD413 and around 1,200 U/LOD for AAC321-1 [Fig. 4B]). Thus, lipase activity in the hexadecane cultures reaches levels more than 10-fold higher than those for above-described triolein cultures. Moreover, extracellular lipase activity in the hexadecane cultures remains highly stable, even after several days of incubation (Fig. 4B). As expected, AAC320-1 does not produce measurable amounts of lipase.

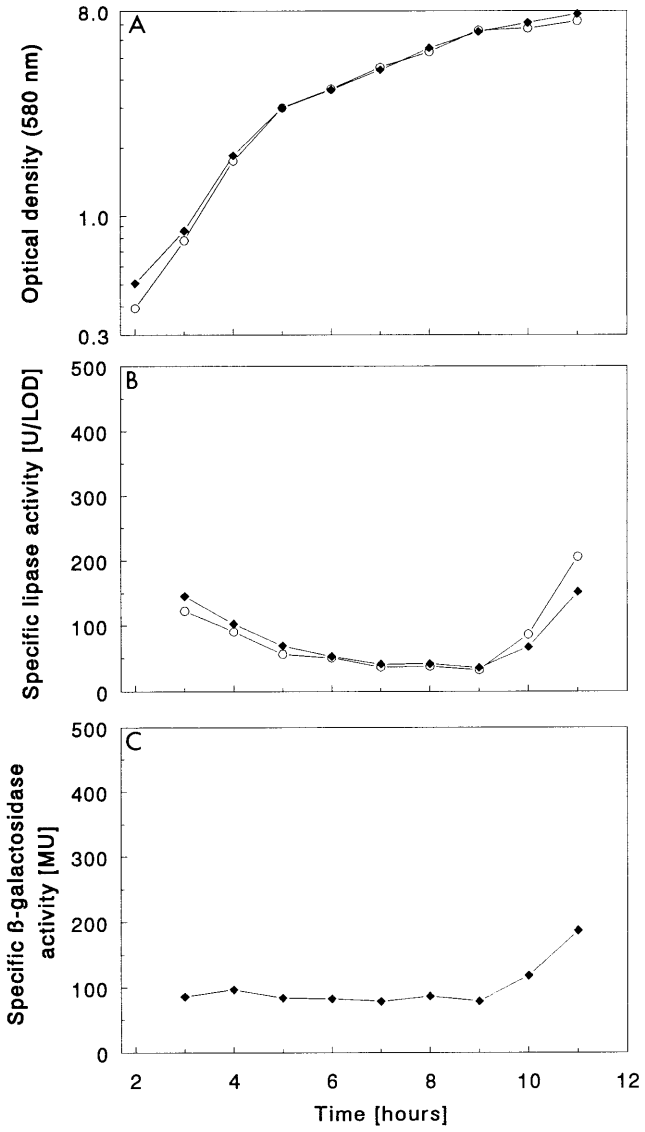

FIG. 3. Growth rate (A), extracellular lipase production (B), and *lipA* expression (measured by the production of β-galactosidase; C) during growth of *A. calcoaceticus* BD413 (○), and AAC321-1 (◆) grown in a mineral medium with triolein as the sole carbon and energy source. For further details, see text. MU, Miller units.

Expression of *lipA* (production of β-galactosidase) is specifically induced toward the early stationary phase during growth of AAC320-1 and AAC321-1 in the hexadecane medium (Fig. 4C). From a very low level of *lipA*::*lacZ* expression during exponential growth (t = 6 h), β-galactosidase levels reach high levels after the onset of the stationary phase. After 26 h, no further *lipA* expression is observed in AAC320-1 and AAC321-1, concurrent with the pattern of appearance of extracellular lipase. This result indicates that lipase production in

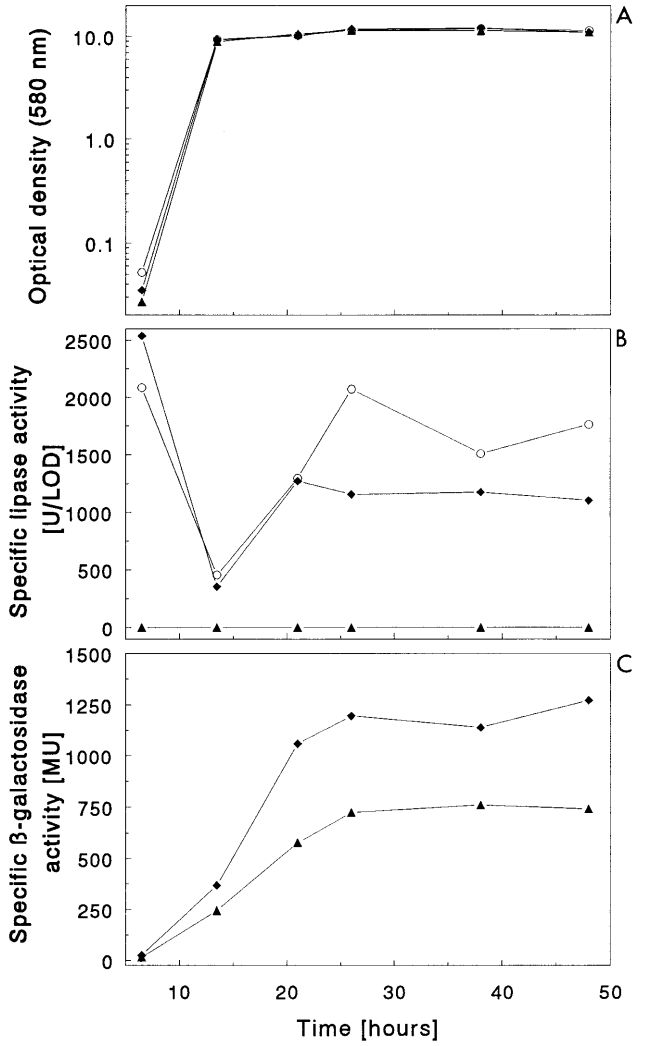

FIG. 4. Growth rate (A), extracellular lipase production (B), and *lipA* expression (C) during growth of *A. calcoaceticus* BD413 (○), AAC320-1 (▲), and AAC321-1 (◆) grown in a mineral medium with hexadecane as the sole carbon and energy source. For further details, see text. MU, Miller units.

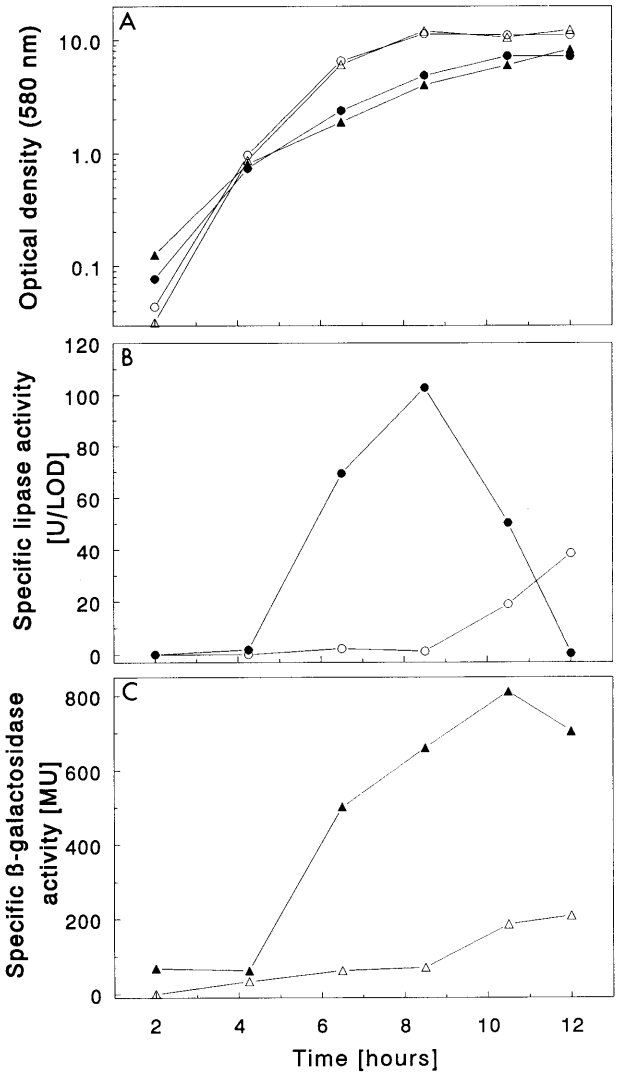

FIG. 5. Growth rate (A), extracellular lipase production (B), and *lipA* expression (C) during growth of *A. calcoaceticus* BD413 (circles) and AAC320-1 (triangles) grown in a mineral medium with 110 mM lactic acid and oleic acid added at 1 mM (filled symbols) or 10 mM (open symbols). MU, Miller units.

these cultures is primarily regulated at the level of *lipA* expression.

Since lipase production in BD413 and AAC321-1 and *lipA* expression in AAC320-1 and AAC321-1 are mainly induced after exponential growth has ceased, hexadecane itself is not likely to be an inducer of lipase production in *A. calcoaceticus*. Rather, it seems that hexadecane, or one of its degradation products, represses *lipA* expression, similar to the situation found during growth on triolein. Nevertheless, levels of *lipA* expression and extracellular lipase activity in the triolein cultures never reached those observed in hexadecane cultures, suggesting that the proposed repressing effect is more easily overcome during growth on the long-chain alkane.

Expression of *lipA* is repressed by long-chain fatty acids. Strong repression of *lipA* expression during growth on triolein and hexadecane suggested that degradation products of these compounds repress the production of extracellular lipase. Therefore, we investigated whether fatty acids, released during catabolism of either the triglyceride and/or the alkane, could repress lipase production and *lipA* expression. Indeed, a strong repressing effect of the long-chain fatty acids hexadecanoic acid (intermediate in hexadecane degradation) and oleic acid (released upon hydrolysis of triolein) on lipase production was initially observed in NB cultures of the wild-type strain supplemented with 17 mM either fatty acid. As suggested by the pattern of *lipA* expression during growth on triolein (Fig. 3C) and hexadecane (Fig. 4C), the mechanism underlying the effect of the added fatty acid could well be the repression of *lipA* expression. This was further investigated through comparison of lipase production levels in BD413 with *lipA* expression levels in AAC320-1. Both strains were grown in a mineral lactic acid medium supplemented with either 1 or 10 mM oleic acid (Fig. 5).

For both strains, growth in the presence of 10 mM oleic acid yields significantly higher growth rates and higher biomass levels compared with growth in the culture supplemented with 1 mM oleic acid (Fig. 5A). During logarithmic growth of the wild type in the 10 mM oleic acid culture, lipase production is strongly repressed, and only after growth has ceased com-

TABLE 2. Expression levels of *lipA* in *A. calcoaceticus* AAC320-1 as a function of the oleic acid concentration during growth[a]

Initial oleic acid concn (mM)	Culture OD_{580}[b]	β-Galactosidase sp act (Miller units)
0.2	0.21	1,061
0.5	0.27	757
1.0	0.33	390

[a] *A. calcoaceticus* AAC320-1 was grown in a mineral lactic acid (28 mM) medium supplemented with various concentrations of oleic acid. Cultures were inoculated ($t = 0$) at equal initial OD_{580}s of 0.07 to 0.09, and growth (OD_{580}) and β-galactosidase specific activity of the cells were recorded for exponentially growing cells at $t = 4.5$ h.

[b] Growth rates of AAC320-1 in 0.2, 0.5, and 1.0 mM oleic acid cultures were 0.69, 0.71, and 0.90 h^{-1}, respectively.

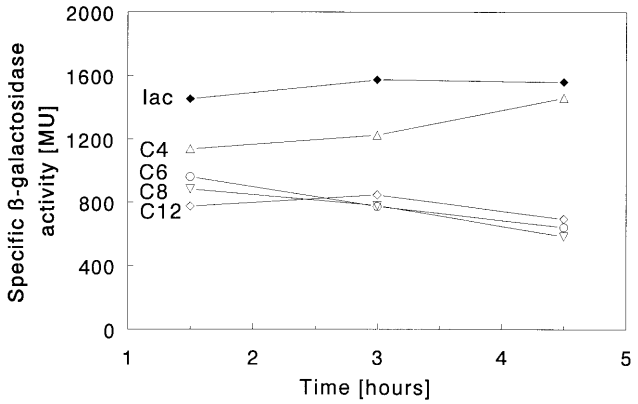

FIG. 6. Effect of short- and medium-chain fatty acids on the level of expression of *lipA*. Cellular levels of β-galactosidase were measured during exponential growth of *A. calcoaceticus* AAC320-1 in mineral medium with 110 mM lactic acid and 3 mM butyric acid (C_4; △), caprylic acid (C_6; ○), caproic acid (C_8; ▽), or lauric acid (C_{12}; ◇). In the control culture, AAC320-1 was grown in mineral medium containing lactic acid as the sole carbon and energy source (◆). MU, Miller units.

pletely, a very weak induction of lipase production is observed (Fig. 5B). In the 1 mM oleic acid culture, however, lipase production is already obtained much earlier during growth and reaches a maximal level of approximately 100 U/LOD, as normally observed in the lactic acid-containing medium (data not shown). Despite this clear burst of lipase activity, the maximal level obtained is relatively low and reminiscent of the lipase levels in triolein-grown cultures (Fig. 3B). In the 1 mM oleic acid culture of BD413, rapid disappearance of lipase activity is observed after an initial peak, even though *lipA* is still being expressed (as judged by the high level of specific β-galactosidase activity in the corresponding culture of AAC320-1, long after lipase activity has dissipated [Fig. 5C]). This presumably is the result of proteolytic degradation of the extracellular lipase, as is discussed below.

Measuring the production of β-galactosidase in AAC320-1 clearly shows that decreased lipase production by BD413 in the oleic acid-containing cultures is caused by repression of expression of *lipA* (Fig. 5C). Only very low levels of β-galactosidase are formed in the 10 mM oleic acid culture of AAC320-1. A weak induction of *lipA* expression may be observable in the stationary phase, presumably after consumption of the fatty acid, which is in line with the production of low amounts of lipase at this stage in growth of BD413 (compare Fig. 5C and B). In contrast, a strong induction of *lipA* expression is observed in the 1 mM oleic acid culture, perfectly reflected in the timing of extracellular lipase formation. Despite the low levels of lipase activity in the extracellular medium, the β-galactosidase levels obtained in the latter culture are comparable to *lipA* expression levels in cultures grown on hexadecane. This strong discrepancy may be caused (at least partly) by lipase inactivation, as will be discussed below.

Since oleic acid is rapidly consumed during growth of the *Acinetobacter* strains in the lactic acid- and oleic acid-containing medium, an accurate quantitative measure of the repressing effect of fatty acids on *lipA* expression cannot be derived from measurements of β-galactosidase activity in AAC320-1. However, it could be determined that an oleic acid concentration of 0.5 mM significantly represses *lipA* expression during the first few hours of growth in a batch culture (Table 2).

We also tested whether short- and medium-chain fatty acids (containing between 4 and 12 carbon atoms) led to repressed expression of *lipA*, as was found for the long-chain fatty acids described above. Expression of *lipA* was again measured in *A. calcoaceticus* AAC320-1 grown in a mineral lactic acid-containing medium supplemented with 3 mM either of these fatty acids (Fig. 6). Of the fatty acids tested, butyrate is the least effective in repressing expression of *lipA*, although during the first 3 h of growth, β-galactosidase levels were significantly lower in the tributyrin cultures (78%) than in the control culture without fatty acids added. Interestingly, caprylic (C_6), caproic (C_8), and lauric (C_{12}) acids are equally effective repressors of expression of the lipase-encoding gene (Fig. 6), with *lipA* expression levels of around 60, 50, and 40% of the control culture at $t = 1.5$, 3, and 4.5 h, respectively. Though these short- to medium-chain fatty acids apparently are more effective repressors than butyrate, they are clearly less effective than shown for oleic acid in Fig. 5.

Apart from an effect on *lipA* expression, the added fatty acids also had a small but noticeable effect on the growth rate of *A. calcoaceticus*, presumably as a result of an uncoupling effect (12). Gradual dissipation of the proton gradient of cells of AAC320-1, by addition of various amounts of dinitrophenol to cells in lactic acid medium, did not lead to a noticeable reduction in the *lipA* expression level, even when the growth rate was almost reduced to zero (at a dinitrophenol concentration in the culture of ≥25 μM [data not shown]). This finding indicates that the fatty acids tested in Fig. 5 and 6 have a direct repressing effect on expression of *lipA*.

Instability of the extracellular lipase and proteolytic degradation in NB cultures. While monitoring extracellular lipase activity during growth of *A. calcoaceticus* BD413 in different media, we have often encountered either low levels of lipase throughout growth (for instance, in cultures with triolein or lactic acid as the sole carbon and energy source [see above]) and/or a strong decrease of maximum activity upon prolonged incubation of the cultures (for instance, in NB cultures, in which specific lipase activity is reduced to almost nondetectable levels within hours after having reached a maximum at the onset of the stationary phase of growth [22]). The latter phenomenon suggested that the extracellular lipase is inactivated in the culture supernatant. To gain further information about this instability, lipase inactivation was studied in vitro.

First, the effect of incubation temperature on the stability of lipase activity in supernatants of NB cultures was investigated (Fig. 7). Lipase activity in these samples declines rapidly during incubation at or above 20°C, whereas the activity is stable at 4°C. At 50°C, the lipase is highly unstable; lipase activity is lost within several hours. Addition of the serine protease inhibitor phenylmethyl sulfonyl fluoride (PMSF) during incubation at 30°C resulted in a marked increase in lipase stability (Fig. 7). This result is in agreement with the observation that addition

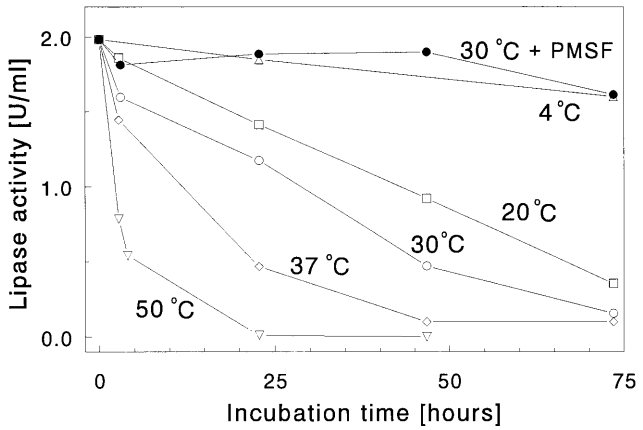

FIG. 7. Stability of extracellular lipase activity in supernatant of an early-stationary-phase culture of *A. calcoaceticus* BD413 grown in NB medium. Lipase activity was measured during incubation at various temperatures indicated. In addition, the effect of 1 mM PMSF during incubation at 30°C was determined (●).

of PMSF prior to the hydrophobic-interaction chromatography steps in the procedure used for purification of the lipase (23) enhanced lipase recovery, whereas PMSF did not affect the activity of the lipase itself.

Although denaturation of the protein may play a role in the loss of lipase activity at 50°C, the foregoing observations suggest that serine protease activity causes inactivation of the lipase in NB cultures. To determine whether the extracellular enzyme was in fact proteolytically degraded in these cultures, supernatants were collected at different stages during growth and concentrated (Fig. 8). Proteins in these samples were separated via SDS-PAGE and blotted onto nitrocellulose (see Materials and Methods). In an immunodetection assay, using polyclonal antibodies raised against the lipase, it could be shown that the lipase protein indeed disappeared from the medium, concurrent with loss of lipase activity in the NB culture (Fig. 8, insert). Moreover, addition of proteinase K to these supernatants showed that the lipase produced by *A. calcoaceticus* BD413 is highly sensitive to proteolysis. After addition of proteinase K (10 μg/ml, incubation at 30°C), all lipase activity was lost within 10 min (not shown). These results strongly suggest that protease activity is responsible for inactivation of the lipase.

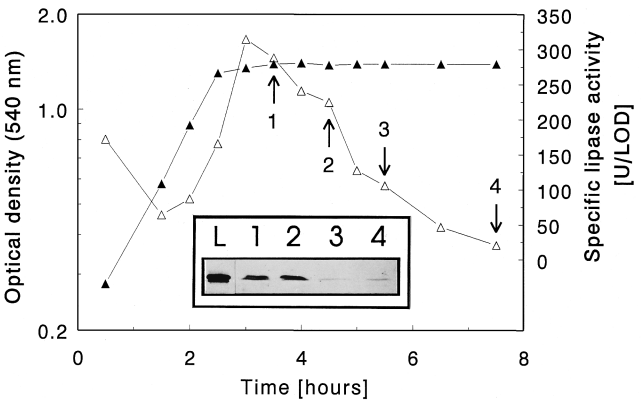

FIG. 8. Degradation of the extracellular lipase of *A. calcoaceticus* BD413 during stationary phase in a batch culture. Growth (▲) and extracellular lipase activity (△) in a culture growing on NB were recorded during exponential growth and the stationary phase. Samples collected at intervals during the stationary phase (as indicated by the numbered arrows) were centrifuged, and their supernatants were concentrated (see Materials and Methods), subjected to SDS-PAGE, and blotted onto a nitrocellulose membrane. LipA was specifically detected by using polyclonal rabbit antilipase antibodies (insert). Numbers in the insert refer to sample numbers; L represents a control with the purified lipase.

DISCUSSION

A mechanistic model for repression of expression of *A. calcoaceticus lipA* by fatty acids. Our results show that lipase production in *A. calcoaceticus* BD413 is strongly repressed by fatty acids, especially long-chain fatty acids. As shown in studies with *lipA*::*lacZ* fusion strain AAC320-1 in lactic acid-containing media, this fatty acid effect is exerted at the level of expression of *lipA*. As soon as repression of *lipA* expression is relieved, extracellular lipase is produced, indicating a strictly genetic control of lipase production under these growth conditions. Similar results have been obtained in a mineral medium with ethanol as the main carbon and energy source (data not shown). This strong fatty acid-dependent repression also explains the patterns of expression of *lipA* during growth of strains AAC320-1 and AAC321-1 in mineral media with triolein or hexadecane.

Repression of lipase production by fatty acids has been detected in several other organisms. Gilbert et al. (8) observed a strong repression of lipase production by fatty acids in *Pseudomonas aeruginosa*. Similar results have been obtained by Chander et al. (1a) for lipase production in *Streptococcus faecalis*. In both organisms, an inverse relationship was found between lipase production and the concentration of long-chain fatty acids in the growth media. This finding indicates that repression of lipase formation by fatty acids may be a common theme in various microorganisms. The significance of this observation, together with the proteolytic instability of the lipase, is further discussed below. The data presented above for *A. calcoaceticus* for the first time reveal the level at which fatty acids repress lipase production.

Mechanistically, the observed repression of *lipA* expression by fatty acids may well involve a regulatory protein that influences transcription of the *lipBA* operon, possibly through binding to the *lipB*-upstream region. We therefore propose that a regulator, which we tentatively designate LipR, mediates repression of transcription of *lipA* in response to binding of a fatty acid (Fig. 9). The fatty acid may stem from LipA-dependent hydrolysis of fats and oils, for instance, triolein, and may be taken up by the cell through a specialized uptake system (28). Thus, the cell would be able to accurately adapt the expression of *lipBA*, and thereby production of lipase, in response to the lipolytic activity already present outside the cell (Fig. 9).

The proposed effect of fatty acids on the activity of a transcriptional regulator is reminiscent of the action of FadR, the central regulator of fatty acid synthesis and catabolism in *E. coli* (for a review, see reference 28). FadR negatively regulates expression of the *fad* regulon, which encodes proteins required for transport, activation, and β-oxidation of long- and medium-chain fatty acids. Interestingly, FadR also activates expression of *fabA*, which encodes an enzyme involved in fatty acid biosynthesis (3, 10, 11). In both cases, it is believed that binding of FadR to its operator site is relieved upon binding long-chain fatty acids or their coenzyme A thioesters. Thus, the regulator would mediate derepression of fatty acid catabolism and deactivation of fatty acid biosynthesis. Recently, Quail et al. (30) have identified another fatty acyl-responsive transcriptional regulator of *E. coli*, designated FarR, with a coeffector specificity and a regulatory mechanism seemingly similar to those of

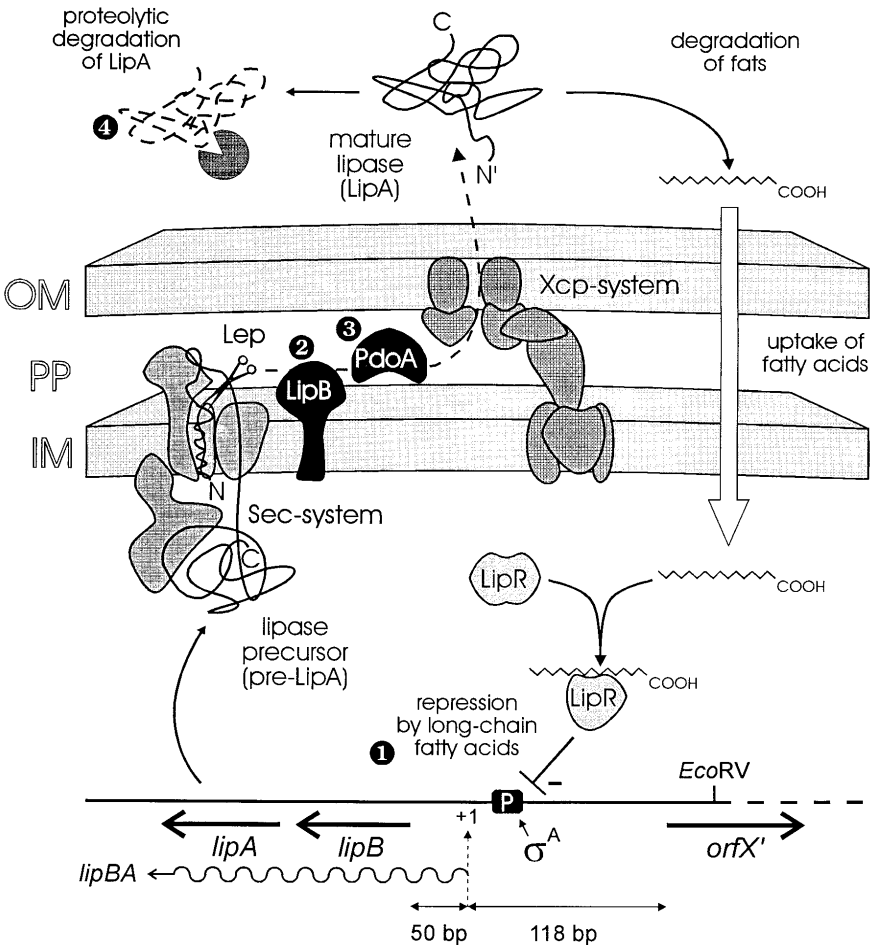

FIG. 9. Model of the steps involved in production of extracellular lipase in *A. calcoaceticus* BD413. Four steps in this production scheme, essential for either lipase production or its regulation, are numbered. 1, regulation of expression of *lipBA*. Promoter (P) recognition by vegetative sigma factor σ^A (24) and fatty acyl-dependent repression of expression (explained in detail in the text) are indicated. LipR denotes the hypothetical regulator of *lipBA* expression which mediates repression (denoted as a minus sign) of *lipBA* transcription upon binding a fatty acid. The fatty acid may originate from LipA-dependent hydrolysis of fats such as triolein. *orfX'* encodes an as yet unidentified protein (24). 2, processing of the lipase precursor by lipase-specific chaperone LipB in the periplasmic space (PP) (24). 3, disulfide bridge formation in LipA by periplasmic protein disulfide oxidoreductase (PdoA [23]). The components of the two independent transport systems, the Sec system (including the leader peptidase [Lep; scissors]) and the Xcp system, responsible for translocation of the lipase across the inner membrane (IM) and the outer membrane (OM), respectively, are indicated (23, 24). 4, proteolytic degradation of LipA in the extracellular medium.

FadR, as judged from mobility shift electrophoresis experiments with addition of fatty acids (10, 30).

The involvement of a regulatory protein in the expression of the *A. calcoaceticus lipBA* operon is presently under investigation. Determination of the nature of the fatty acid-responsive behavior of LipR, whether through a classical repression mechanism or through deactivation as proposed for FadR/FarR, will obviously have to await identification of LipR itself. The latter mechanism would even imply a role for LipR in activation of transcription of *lipBA* in the absence of fatty acids. In this case, the operator site would be expected to be located upstream of the *lipBA* promoter region (2), such as found for the FadR binding site upstream of the *E. coli fabA* gene (10). Interestingly, the 145-bp intergenic region between *orfX'* and *lipB* carries unusually AT-rich regions with several poly(A) and poly(T) tracts (24). As determined for *E. coli* FarR (30), these sequences may form a binding site(s) for the putative LipR.

Fatty acid-dependent repression of lipase production and LipA inactivation as physiological mechanisms controlling the amount of active extracellular lipase. LipA is required for growth of *A. calcoaceticus* BD413 on triolein. Nevertheless, only low levels of lipase activity are found in triolein-grown cultures, as a result of strong repression of *lipA* expression by the liberated oleic acid. Apparently, lipase levels need to be kept low under these conditions, even though growth can proceed only by virtue of the activity of LipA. The resulting physiological competition between expression and repression of *lipA* strongly suggests that production of the extracellular lipase has both beneficial and deleterious effects. The latter negative effect may be caused by the uncoupling effects of fatty acids (12). Especially fatty acids with medium chain length would be potent in this respect (12, 37). It is tempting to speculate that such toxicity has even provided the selection pressure for the evolution of the fatty acid-dependent repression mechanism of *lipA* expression. In triolein media, the compromise apparently results in a low level of lipase production, ensuring only very low concentrations of active extracellular lipase activity, just enough to support growth.

In several studies of lipase production in pseudomonads, olive oil (triolein) has been found to be an optimal substrate for lipase production. For example, Shimizu and coworkers

(16, 34) described optimum conditions for lipase production by *Pseudomonas fluorescens* during growth on olive oil as the sole carbon source. Also for *P. aeruginosa* (8) and *P. glumae* (6), olive oil was found to strongly stimulate lipase production. Significantly, in all of these examples, strains were grown in fed-batch cultures in which the olive oil concentration was kept low. This finding indicates that low levels of lipase substrate induce lipase production in these organisms, consistent with the results presented above.

Repression of the formation of LipA by fatty acids in *A. calcoaceticus* BD413 does not seem to be the only mechanism that negatively regulates extracellular lipase activity. LipA is also subject to strong inactivation (Fig. 9). This has clearly been demonstrated in NB medium. LipA degradation may account for the strong discrepancy between the high levels of expression of *lipA* and the low levels of extracellular lipase activity that appear in the lactic acid medium. The latter has also been observed during growth on ethanol (not shown). In addition, during growth on Tween 80 (polyoxysorbitan monooleate) as the carbon and energy source, lipase activity is rapidly lost a few hours after a maximum has been reached, reminiscent of the pattern of lipase activity in NB medium (data not shown). Only in the medium with hexadecane, LipA activity is stabilized and may therefore reach relatively high levels. The latter assumption is based on the observation that expression of the *lipA* gene (after the fatty acid-dependent repression of *lipA* expression has been relieved) is not elevated in the mineral hexadecane medium, in comparison with the cultures with lactic acid, while lipase activity reaches 10- to 20-times-higher levels during growth on the long-chain alkane. Addition of an emulsion of hexadecane and arabic gum to NB culture supernatants also results in enhanced stability of lipase activity (1).

Figures 7 and 8 show that an extracellular serine protease is responsible for degradation of the lipase in NB supernatants. This observation is in striking contrast with the situation described by Stuer et al. (32), who reported that the extracellular lipase produced by *P. aeruginosa* is tightly bound to lipopolysaccharide (LPS) released from the cell. This association conferred a remarkable stability to the enzyme with respect to proteolytic degradation. In cell-free culture supernatants, lipase was stable at room temperature for weeks and highly resistant to treatment with proteinase K. In contrast, the lipase of *A. calcoaceticus* is highly sensitive to degradation by proteinase K, indicating that this lipase is not protected against proteolysis via association with LPS. No protease activity could be detected in culture supernatants of *A. calcoaceticus* BD413 grown in NB medium, as tested via degradation of resorufin-labeled casein in a sensitive protease assay (Boehringer Mannheim) (not shown). However, production of protease activity recently has been detected in concentrated supernatants of cultures of *A. calcoaceticus* BD413, via activity staining in polyacrylamide gels, under nondenaturing conditions (27a).

A. calcoaceticus BD413 apparently produces a lipase which is strongly sensitive to degradation by one of its own protease(s). Such protease sensitivity has also been reported for several lipases produced by other microorganisms, such as *P. glumae* (7) and the fungus *Rhizopus delemar* (17), and is a recognized problem in the large-scale production of lipases in industry (7, 17). Since protease activity in *A. calcoaceticus* BD413 seems responsible for lipase inactivation in most of the media described (presumably all except the hexadecane medium), lipase production apparently often takes place when protease activity is already present or protease production is also induced. Together with the apparent sensitivity of *A. calcoaceticus* LipA towards the endogenous protease, this observation suggests that proteolytic inactivation of the lipase has a physiological role in regulation of the amount of active extracellular LipA (Fig. 9). Perhaps prolonged lipase activity is detrimental to the cell under many physiological conditions, even when fatty acids are not the major problem. In the presence of an emulsion of the nonlipase substrate hexadecane, the lipase may simply be protected against proteolytic degradation, possibly as a result of hydrophobic interactions between LipA and hexadecane micelles.

In conclusion, lipase production in *A. calcoaceticus* is regulated at the genetic level through expression of the *lipA* gene, and the level of active extracellular LipA may be influenced at the level of proteolytic stability or instability of the lipase. Furthermore, as previously determined, the action of lipase chaperone LipB (24) and S-S bridge formation by the periplasmic processing enzyme PdoA (23) strongly affect lipase production (Fig. 9). The entire process of production of lipase apparently entails multiple cellular and possibly extracellular layers of control. In combining further systematic physiological and genetic studies, we aim to obtain a detailed quantitative understanding of the regulation of the production of extracellular lipases in gram-negative bacteria such as *A. calcoaceticus.*

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