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Characterization of Lipase-Deficient Mutants of Acinetobacter calcoaceticus BD413: Identification of a Periplasmic Lipase Chaperone Essential for the Production of Extracellular Lipase

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Acinetobacter calcoaceticus BD413 produces an extracellular lipase, which is encoded by the lipA gene. Five lipase-deficient mutants have been generated via random insertion mutagenesis. Phenotypic characterization of these mutants revealed the presence of as many as four lipolytic enzymes in A. calcoaceticus. Biochemical evidence classified four of the mutants as export mutants, which presumably are defective in translocation of the lipase across the outer membrane. The additional mutant, designated AAC302, displays a LipA+ phenotype, and yet the mutation in this strain was localized 0.84 kb upstream of lipA. Sequence analysis of this region revealed an open reading frame, designated lipB, that is disrupted in AAC302. The protein encoded by this open reading frame shows extensive similarity to a chaperone-like helper protein of several pseudomonads, required for the production of extracellular lipase. Via complementation of AAC302 with a functional extra- chromosomal copy of lipA, it could be determined that LipB is essential for lipase production. As shown by the use of a translational LipB-PhoA fusion construct, the C-terminal part of LipB of A. calcoaceticus BD413 is located outside the cytoplasm. Sequence analysis further strongly suggests that A. calcoaceticus LipB is N terminally anchored in the cytoplasmic membrane. Therefore, analogous to the situation in Pseudomonas species, the lipase helper protein presumably is active in the periplasm. In contrast to the situation in Pseudomonas species, however, lipB in A. calcoaceticus is located upstream of the structural lipase gene. lipB and lipA form a bicistronic operon, and the two genes are cotranscribed from an Escherichia coli σ70-type promoter. The reversed order of genes, in comparison with the situation in Pseudomonas species, suggests that LipA and LipB are produced in equimolar amounts. Therefore, the helper protein presumably does not only have a catalytic function, e.g., in folding of the lipase, but is also likely to act as a lipase-specific chaperone. A detailed model of the export route of the lipase of A. calcoaceticus BD413 is proposed.

At the onset of the stationary phase in rich media, the gram-negative soil bacterium Acinetobacter calcoaceticus BD413 produces several lipolytic enzymes (40), which have partly overlapping substrate specificities. BD413 estA, encoding one of the cell-bound esterases (40), and lipA, encoding the extracellular lipase (43), have been cloned and characterized. The regulation of their expression is currently being investigated. In addition, we also aim at defining posttranscriptional factors involved in regulation of enzyme production. The latter aspect is especially important for the extracellular lipase of A. calcoaceticus BD413, since several components involved in export of the protein, including concurrent protein processing events, may have significant control over lipase production.

The extracellular lipase of A. calcoaceticus BD413 presumably is exported from the cell via a two-step translocation process (43): across the cytoplasmic membrane, this translocation will be mediated by the Sec system (59, 66), whereas translocation through the outer membrane proceeds via a translocation complex similar to the Xcp system in Pseudomonas spp. (69) and the Pul system in Klebsiella spp. (59). These assumptions are based upon the identification of an N-terminal export signal peptide, indicative of the involvement of a Sec-like system, and upon the high degree of similarity of A. calcoaceticus LipA to Pseudomonas lipases that are secreted via a two-step mechanism. Moreover, we have demonstrated that a periplasmic processing enzyme, a protein disulfide oxidoreductase, is required for high-level production of lipase in A. calcoaceticus BD413 (43).

In Pseudomonas spp., production of lipases that are similar to Acinetobacter LipA requires the presence of a lipase-specific helper protein, which is N terminally anchored in the cytoplasmic membrane, with its C-terminal domain in the periplasm. Activity of this type of protein is essential for the second lipase translocation step, across the outer membrane (14, 34). In the periplasm, such helper proteins have been shown to mediate the formation of secondary structure elements in the periplasmic form of the lipase, suggesting a chaperone-like function of the helper protein. Unlike the situation in pseudomonads, as yet no essential lipase helper protein has been identified in A. calcoaceticus. In Pseudomonas species, these proteins are invariably encoded downstream of the structural lipase gene, and this is not the case in A. calcoaceticus BD413 (43).

Previously, we have reported on the identification of two
lipase-deficient mutants of *A. calcoaceticus* BD413, generated via random insertion of a kanamycin resistance marker (74). In this paper, these mutants, including three additionally generated mutant strains, are phenotypically characterized. Biochemical evidence obtained from four pleiotropic mutants strongly suggests the presence of an Xcp-like protein export system in *A. calcoaceticus* BD413 and its requirement for export of the lipase. Genetic characterization of an additional mutant demonstrates that lipase production in *A. calcoaceticus* also requires a periplasmic lipase-specific helper protein that is membrane bound. Our results suggest a dedicated chaperone function for this protein, as proposed for *Pseudomonas cepacia* by Hobson et al. (27). Consequently, the helper protein will be referred to as a chaperone.

### MATERIALS AND METHODS

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

**Media and culture conditions.** Strains of *A. calcoaceticus* and *Escherichia coli* were grown in LB medium (5 g of NaCl, 5 g of yeast extract [Difco], and 10 g of Bacto Tryptone [Difco] per liter [pH 7.4]) or nutrient broth (N broth [Gibco]; 8 or 16 g/liter), either in Erlenmeyer flasks on a gyratory shaker or in MultiGen fermentor vessels (New Brunswick Scientific Co.), with saturating aeration at 30°C (*A. calcoaceticus*) or 37°C (*E. coli*). When appropriate, the optical density of the cultures, measured at 540 nm, was recorded as a representation of growth. For plates, the above liquid media were solidified with 1.5% (wt/vol) agar. The bacterial strains and plasmids used are listed in Table 1.

### TABLE 1. Strains, plasmids, and phages used in this study

<table>
<thead>
<tr>
<th>Strain, phage, or plasmid</th>
<th>Relevant characteristics</th>
<th>Marker(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
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<td></td>
</tr>
<tr>
<td>JM83</td>
<td>Δlac(proAB) thi</td>
<td>(880 lacZΔM15)</td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>ΔlacU169 (880 lacZΔM15) recA1 thi-1 relA1</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>TG1</td>
<td>supE hsdS3 Δ5 thi Δ(lac-proAB) F' [traD36 proAB+ lacF' lacZΔM15]</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td><em>A. calcoaceticus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD413</td>
<td>Wild type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD413-iv10</td>
<td>Auxotrophic for Ile, Leu, and Val</td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>AAC300</td>
<td>BD413 (xcpII), lipase deficient (xcp)</td>
<td>Km'</td>
<td>74</td>
</tr>
<tr>
<td>AAC302</td>
<td>BD413 (xcpII), lipase deficient (lipBA)</td>
<td>Km'</td>
<td>74</td>
</tr>
<tr>
<td>AAC302rec</td>
<td>AAC302 recA::Cm</td>
<td>Km' Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>AAC303</td>
<td>BD413 (xcpII), lipase deficient (xcp)</td>
<td>Km'</td>
<td>This study</td>
</tr>
<tr>
<td>AAC304</td>
<td>BD413 (xcpII), lipase deficient (xcp)</td>
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<td>This study</td>
</tr>
<tr>
<td>AAC305</td>
<td>BD413 (xcpII), lipase deficient (xcp)</td>
<td>Km'</td>
<td>This study</td>
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<tr>
<td>AAC316</td>
<td>BD413 (lipA:: Ser-99 to Ala)</td>
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<td>43</td>
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<td>AAC320-1</td>
<td>BD413 (lipA::lacZ-Km)</td>
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<td>AAC320-1 recA::Cm</td>
<td>Km' Cm'</td>
<td>This study</td>
</tr>
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<td>AAC324</td>
<td>BD413 (lipR::pALJA26)</td>
<td>Amp'</td>
<td>This study</td>
</tr>
<tr>
<td>AAC342</td>
<td>AAC316 (Δ4.3 kbp estA EcoRI)::(3.5 kbp Cm, EcoRI)</td>
<td>Cm'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Phages</strong></td>
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</tr>
<tr>
<td>M13mp18</td>
<td>αlacZ</td>
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<td>77</td>
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<tr>
<td>M13mp19</td>
<td>αlacZ</td>
<td></td>
<td>77</td>
</tr>
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<td>mpALJA2</td>
<td>M13mp19 BamHI::lipBA (2.4 kbp, Sau3A')</td>
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<td>43</td>
</tr>
<tr>
<td>mpALJA2b</td>
<td>M13mp18 BamHI::lipBA (2.4 kbp, Sau3A')</td>
<td></td>
<td>43</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pHA1</td>
<td>pWH1266 (∆BR322, PvuII::pUN121, PstI)</td>
<td>Amp' Te'</td>
<td>This study</td>
</tr>
<tr>
<td>pAKA302</td>
<td>pMTL24p BamHI::(6.5 kbp AAC302 DNA, BclI, nptII)</td>
<td>Amp' Km'</td>
<td>This study</td>
</tr>
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<td>pAKA1024-32</td>
<td>BD413 DNA, ΔεσA::Cm</td>
<td>Amp' Cm'</td>
<td>40</td>
</tr>
<tr>
<td>pALJA25-1</td>
<td>pHA1::lipB (5.8 kbp BD413 DNA, HindIII)</td>
<td>Amp' Te'</td>
<td>This study</td>
</tr>
<tr>
<td>pALJA26</td>
<td>pPH07::lipB (5.8 kbp BD413 DNA, HindIII) LipB::PhoA</td>
<td>Amp'</td>
<td>This study</td>
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<td>pALJA27</td>
<td>pWH1274 tet::lipA</td>
<td>Amp'</td>
<td>This study</td>
</tr>
<tr>
<td>pALJA230</td>
<td>pWH1274::lipA</td>
<td>Amp'</td>
<td>This study</td>
</tr>
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<td>pALJA432</td>
<td>pUN121::lipBA</td>
<td>Amp'</td>
<td>43</td>
</tr>
<tr>
<td>pARA0</td>
<td>pUN121::BD413 recA</td>
<td>Amp' Te'</td>
<td>54</td>
</tr>
<tr>
<td>pARA2Cm</td>
<td>pARA0 SaI::(3.5 kbp Cm, SalI)</td>
<td>Amp' Cm' Te'</td>
<td>This study</td>
</tr>
<tr>
<td>pPH07</td>
<td>Promoterless phoA</td>
<td>Amp'</td>
<td>20</td>
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<td>pKT210</td>
<td>IncQ broad-host-range vector, Cm</td>
<td>Cm' Sm'</td>
<td>2</td>
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<tr>
<td>pMTL24p</td>
<td>αlacZ</td>
<td>Amp'</td>
<td>6</td>
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<tr>
<td>pMTL24pCm</td>
<td>Cm</td>
<td>Amp' Cm'</td>
<td>40</td>
</tr>
<tr>
<td>pT11</td>
<td>aphA3</td>
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<td>56</td>
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<td>pUC4K</td>
<td>nptII</td>
<td>Amp' Km'</td>
<td>71</td>
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<td>pUN121</td>
<td>ColEI(TcEI)</td>
<td>Amp' Te'</td>
<td>48</td>
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<td>pWH1266</td>
<td>Acinetobacter-E. coli shuttle vector</td>
<td>Amp' Te'</td>
<td>29</td>
</tr>
<tr>
<td>pWH1274</td>
<td>Acinetobacter-E. coli shuttle vector</td>
<td>Amp' Te'</td>
<td>29</td>
</tr>
</tbody>
</table>

* Te' refers to sensitivity due to an inactive tetracycline resistance gene. nptII, the kanamycin resistance gene of pUC4K; aphA3, the kanamycin resistance gene of pT11. Both can be isolated as a 1.3-kbp BamHI fragment. Cm, 3.5-kbp fragment carrying the chloramphenicol resistance gene of pKT210. These strains possibly carry a mutation(s) in the xcp protein translocation system (xcp) [see text].

The sizes (in kilobase pairs) of relevant DNA fragments and restriction enzymes used for isolation and deletion are indicated.

A. **Δlac(proAB) thi**

B. **ΔlacU169 (880 lacZΔM15) recA1 thi-1 relA1**

C. **Δ(lac-proAB) F' [traD36 proAB+ lacF' lacZΔM15]**

### References

1. Hobson et al. (27).
2. This study.
3. The sizes (in kilobase pairs) of relevant DNA fragments and restriction enzymes used for isolation and deletion are indicated.
Antibiotics were used at the indicated final concentrations, in plates and in liquid media: ampicillin, 200 µg/mL for A. calcoaceticus and 100 µg/mL for E. coli; kanamycin, 15 µg/mL for A. calcoaceticus and 50 µg/mL for E. coli; tetracycline, 15 µg/mL; and chloramphenicol, 50 µg/mL for A. calcoaceticus and 20 µg/mL for E. coli.

Chemicals. Restriction enzymes and T4 DNA ligase were obtained from Pharmacia (Pharmacia Biotech). -32P-dATP (1 C/mm) was purchased from Amersham Life Science. Sequence primers were synthesized by Pharmacia.

Recombinant DNA techniques. Acinetobacter chromosomal DNA was isolated as described by Vosman and Hellingswift (73). Plasmid DNA was purified by the method of Ish-Horowiz and Burke (33) or as described by Del Sal et al. (10). Plasmids were transformed into E. coli as described in reference 47. Introduction of plasmid DNA into A. calcoaceticus BD413 by natural transformation was performed as described by Palm et al. (58). Southern detection of DNA fragments (with nonradioactive digoxigenin-dUTP-labeled probes and the alkaline phosphatase detection system of Boehringer Mannheim) were performed as described previously (40, 74). For primer extension analyses, total RNA was isolated from early-stationary-phase N broth cultures of several strains of A. calcoaceticus (see text and Fig. 6), essentially according to the method of Chirgwin et al. (8). An amount of cells equivalent to 100 ml of a culture with an optical density at 600 nm of 1.0 was used for all isolations. Primer extension analysis of lipB, including the required sequence reactions, was performed as described previously (41). Primer LipB1 (see Fig. 4A) was used for these experiments. All additional recombinant DNA techniques were performed essentially according to the method of Sambrook et al. (63).

Sequence analysis. The nucleotide sequence of A. calcoaceticus chromosomal DNA in mpLA12, mpLA2b, and derivatives of pVA302-1 was determined from both strands by the dyeode chain termination method (65), as described previously (40) with either the M13 universal primers or custom-synthesized primers. Sequence data were analyzed with GCG (Gene 6.5, Intelligenetics) and the University of Wisconsin Genetics Computer Group software (GCC Package, version 7).

Measurement of lipase activity and protein concentration. Lipase activity in culture supernatants was determined as described for A. calcoaceticus. AAC311-2 carries a sit-directed mutation in the lipA open reading frame (ORF), rendering the lipase inactive (43) (see Table 3). AAC311-2 completely lacks a 4.3-kbp EcoRI fragment which encompasses the complete estA gene and carries a chromosomal promoterless marker instead (40). For construction of the double mutant AAC320-1, the plasmid that was used to introduce the estA mutation in AAC311-2 (pATA1024-32) was transformed into AAC316, and transformants were screened as described for AAC311-2 (40). A. calcoaceticus AAC320 carries the 4.3-kbp EcoRI EstA-encoding fragment, as was verified by Southern hybridizations. In addition, it is lipase deficient, since it produces an inactive extracellular lipase.

Construction of pAHA1 and pALJA25-1. Plasmid pWII1266, an E. coli-chromosomemediated shuttle vector, has been constructed by ligation of a cryptic plasmid of A. calcoaceticus BD413 DNA presented in this paper has been submitted to the database.

To inactivate the lipA gene of AAC202 and AAC320-1, the 4.3-kbp fragment which is in fact the cI fragment presented in this paper has been submitted to the database.

RESULTS

Generation of lipase-deficient mutants of A. calcoaceticus BD413. Previously, we had described the isolation of two lipase-deficient mutants (AAC300 and AAC302) of A. calcoaceticus BD413 (74), hereafter to be called AAC300-ivl and AAC302-ivl, since the auxotrophic strain A. calcoaceticus BD413-ivl10(36) was used as the wild-type strain for mutation (74). The two mutants were generated by random insertion of a kanamycin resistance (nptII) cassette (lacking a replicon [Fig. 1]) and were selected for lipase deficiency on N broth plates containing the (phospho)lipase substrate egg yolk (74).
The mutagenesis procedure makes use of the high level of competence for natural transformation of *A. calcoaceticus* BD413 (and its derivatives [37, 53]). Homologous recombination events lead to insertion of the antibiotic marker into the chromosome, which can result in disruption of wild-type sequences (Fig. 1) (see also the work of Vosman et al. [74]). This procedure allows for easy identification of the mutated chromosomal region, e.g., by recovery of the marker in subsequent cloning steps (54).

Via a similar procedure, but now with the isolated kanamycin resistance gene *aph*4 from plasmid pPJ1 (56), three additional lipase-deficient strains (AAC303-ivl, AAC304-ivl, and AAC305-ivl) were isolated. The total of these five lipase-deficient strains of *A. calcoaceticus* were selected out of approximately 21,000 kanamycin-resistant transformants. The mutations in the BD413-ivl10 genetic background finally were transferred to the wild-type strain BD413, via transformation of BD413 with chromosomal DNA of the mutant strains and selection for kanamycin resistance. All resulting strains were lipase negative, as tested on egg yolk plates (see below [Table 2]). Also, no significant extracellular lipase activity could be detected in liquid cultures of these strains in N broth (as judged from the lack of hydrolysis of the LipA substrate p-nitrophenyl palmitate [data not shown]), while wild-type BD413 produces fair amounts of extracellular lipase activity in this medium (40).

Representative lipase-negative strains of BD413, obtained from separate transformations, were designated AAC300, -302, -303, -304, and -305, respectively. These strains were used for further analysis.

Via Southern hybridization of chromosomal digests of all five lipase-deficient mutants, with the appropriate kanamycin resistance marker as a probe, it could be shown that they all contain the marker inserted into a different chromosomal context (reference 74 and present study). This indicates that all mutants have been generated via independent insertion events. However, since different chromosomal fragments may have been deleted in these strains (in the case of marker insertion, via a double-crossover event [Fig. 1] [54]), their lipase-deficient phenotype may be the result of disruption of the same gene. Therefore, different phenotypic screens were applied, in order to distinguish classes of lipase-deficient mutants.

**Identification of two classes of lipase-deficient mutants and four lipolytic enzymes.** To obtain more detailed information on the mutant phenotype of the five strains described above, each strain was qualitatively tested with respect to degradation of four different lipidic substrates in N broth plates (Table 2).

<table>
<thead>
<tr>
<th>Strain (relevant genotype)</th>
<th>Olive oilb</th>
<th>Tributyrinc</th>
<th>Egg yolkd</th>
<th>Tween 80e</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD413 (wild type)</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>AAC302 (lipBA)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AAC300 (xcp?)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AAC303 (xcp?)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AAC304 (xcp?)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AAC305 (xcp?)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AAC316 (lipA)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>AAC311-2 (estA)</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>AAC342 (lipA estA)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
</tbody>
</table>

*See Table 1 and text.

a An orange fluorescent zone (1 to 3 mm) is produced around the colony, after several days of incubation (+ +), or no fluorescent zone is visible (–).
b A large clearance (8 to 15 mm) is visible around the colony, which develops after 1 day of incubation (+ + +), or a small zone of clearance (1 to 3 mm) is formed after several days of incubation (+).
c A large turbid zone (12 to 17 mm) is formed around the colony, after 3 days of incubation (+ + +), or no turbid zone is visible, even after 5 days of incubation (–).
in AAC311-2 has no detectable effect (Table 2). Interestingly, the \textit{estA} deletion in mutant strain AAC342 has no effect on the residual degradation of tributyrin, compared with the lipase-deficient mutants. In fact, from the remaining tributyrin degradative activity in double mutant AAC342, lacking both EstA and LipA activities, it must be concluded that an additional lipolytic enzyme is present in \textit{A. calcoaceticus} BD413. As is clear from Table 2, this third enzyme in the lipolytic system of \textit{A. calcoaceticus} apparently shows no significant activity towards olive oil or egg yolk but is able to degrade tributyrin. The additional LipA-independent tributyrin degradative activity of \textit{A. calcoaceticus} BD413 hereafter will be referred to as tributyrin esterase.

During growth on plates containing the artificial ester Tween 80 (polyoxyethylene sorbitan monooleate) as another potential substrate for the lipolytic enzymes in \textit{A. calcoaceticus}, wild-type BD413 produces a large turbid zone around the colonies (Table 2). Inactivation of the extracellular lipase in AAC316 does not result in a lowering of the capacity for Tween 80 degradation, nor is Tween 80 degradation by AAC302 distinguishable from the wild-type activity. Interestingly, \textit{estA} mutant AAC311-2 and \textit{estA-lipA} double mutant AAC342 also exhibit wild-type degradation levels. This indicates that both the extracellular lipase and the intracellular esterase EstA have no significant in vivo activity towards Tween 80 and that degradation of this substrate by \textit{A. calcoacet-

Identification of this additional esterase activity raises the question of whether or not the Tween esterase is identical to the above-described tributyrin esterase. Though AAC300, -303, -304, and -305 still produce the tributyrin esterase activity, these mutants completely lack halo formation on Tween 80 plates (Table 2). This indicates that the enzymatic activity responsible for these two reactions is caused by two separate enzymes and hence that \textit{A. calcoaceticus} BD413 produces at least four lipolytic enzymes, i.e., LipA, EstA, the tributyrin esterase, and the Tween esterase. The clear difference in lipolytic phenotype, compared with \textit{lipA} mutants AAC316 and AAC342, indicates that lipase-deficient strains AAC300, -303, -304, and -305 are also unable to produce the Tween esterase, which is still present in the former two \textit{lipA} mutants. Apparently, AAC300, -303, -304, and -305 carry a mutation with a pleiotropic effect on the production of two of four lipolytic enzymes of \textit{A. calcoaceticus} BD413 that have been identified thus far. Though the four pleiotropic insertion mutants arose through separate mutational events (see above), their similarity in lipolytic phenotype suggests that they may be disturbed in a similar cellular function. This phenotype clearly represents a different class of insertional mutant compared with AAC302, which shows a true LipA\textsuperscript{-} phenotype (Table 2).

**Involved of an Xcp-like system in the translocation of the lipase and the Tween esterase across the outer membrane.**

Strains AAC300, -303, -304, and -305 may carry regulatory mutations affecting the regulation of the expression of several, but not all, lipolytic activities in \textit{A. calcoaceticus}. Another simple explanation would be that these pleiotropic mutants of \textit{A. calcoaceticus} are impaired in export of extracellular enzymes like LipA, i.e., a component(s) of the proposed Sec-Xcp-like two-step secretion route of the lipase (43) (see also Fig. 8) may be lacking in these mutants. In this case, the complete absence of Tween 80 degradation in mutants AAC300, -303, -304, and -305 could well be explained if the Tween esterase of \textit{A. calcoaceticus} BD413 is also transported out of the cell and exhibits its hydrolytic activity in the extracellular medium or at the outer surface of the cell.

To test the hypothesis that AAC300, -303, -304, and -305 are export mutants, potential accumulation of the LipA protein in the mutant cells was investigated via immunodetection of the LipA protein in cell extracts, using polyclonal rabbit anti-LipA antibodies (43). Although some cross-reactivity is present, even when a preabsorbed fraction of the antibody is used, it can be seen that the lipase protein clearly accumulates in cells of AAC300, -303, -304, and -305, compared with wild-type BD413 (Fig. 2). The lipase was not detectable in supernatants of these cultures, whereas earlier experiments (43) had shown that it does accumulate extracellularly in cultures of BD413. Thus, LipA is formed in all four pleiotropic mutants, arguing against the potential regulatory nature of the mutation in these strains, and yet the lipase protein apparently is not exported to the extracellular medium. No lipase protein is detected in cell extract of AAC302 (Fig. 2, lane 3). This suggests that LipA is not formed at all in this mutant strain (see below).

The immature lipase of \textit{A. calcoaceticus} BD413, because of the presence of the presumed export signal sequence, has a calculated mass of 34.8 kDa (43). As can be concluded from Fig. 2, in cell extracts of BD413 and mutants AAC300, -303, -304, and -305, the accumulated form of the lipase has a molecular mass equivalent to that of the mature (extracellular) lipase (i.e., 32.1 kDa). Apparently, the accumulated lipase protein in these strains has passed the Sec system in the cytoplasmic membrane and is in fact of periplasmic origin. This suggests that the mutation(s) of AAC300, -303, -304, and -305 affect(s) the translocation process of the lipase (and other proteins) across the outer membrane. The same explanation presumably accounts for the lack of production of Tween esterase in these strains (Table 2).

**Complementation of the mutation in \textit{A. calcoaceticus} AAC302.**

Mutant strain AAC302, which showed a true LipA phenotype (see above) (Table 2 and Fig. 2) was further genetically characterized. A complementation experiment was performed, in order to isolate and identify the wild-type DNA corresponding to the disrupted region in AAC302. A gene library was constructed of EcoRI-digested chromosomal DNA fragments of wild-type \textit{A. calcoaceticus} BD413, ligated into the EcoRI site of the broad-host-range vector pKT210. This gene
The nucleotide sequence has been obtained, including the Hin resistance gene (orientation in AAC302 and pAKA302 is unknown; double arrow). Vector fragments are not drawn to scale. Hin Bc scale; indicated by an interruption in the line). The 5.8-kbp fragment of chromosomal DNA of A. calcoaceticus (43), which contains the lipA gene. Recently, we have cloned a 2.4-kbp BcII fragment of chromosomal DNA of A. calcoaceticus BD413 (43), which contains the lipA gene, as determined by sequence analysis of parts of this fragment [in mpALJA2(b) (Fig. 3)]. Restriction analysis and Southern hybridizations on various digests of pAVA302-1, with the lipA region of mpALJA2(b) as a probe, confirmed the presence of the lipA gene on the insert in pAVA302-1 (not shown).

AAC302 carries a mutation upstream of the lipA gene. To determine whether lipase-deficient A. calcoaceticus AAC302 carries a mutation in the structural lipase gene lipA, the inserted kanamycin resistance marker and bordering chromosomal sequences were cloned for analysis of the mutated region. Previously, we have shown that the nptII gene is located on a 6.5-kbp BcII fragment on the chromosome of AAC302 (74). Therefore, chromosomal BcII fragments of AAC302 of approximately 6 to 7 kbp were cloned into BamHI-linearized PMTL24p(6) in E. coli DH5α, and transformants were screened for kanamycin resistance. Via restriction analysis of the resulting plasmid, designated pAKA302 (Fig. 3), and through Southern hybridization with different fragments of mpALJA2(b) as a probe, it was found that the nptII gene is not located within the lipA gene on the AAC302 chromosome. However, it was found that the mutant locus of AAC302 is located 0.84 kbp upstream of the lipase structural gene (Fig. 3). This indicated disruption of another ORF in AAC302, which is involved in the expression or processing of the lipase in wild-type A. calcoaceticus BD413. Alternatively, the upstream mutation could have a polar effect on expression of lipA (see below).

Identification of a gene encoding a lipase chaperone upstream of the lipA gene. The above-described complementation results prompted us to further determine the nucleotide sequence of the fragment upstream of the lipA gene on the BD413 chromosome (the sequence of the 2.055-bp HindIII-BcII fragment encompassing the complete lipA ORF has been described elsewhere [43]). By using inserts of pAVA302-1 and mpALJA2(b) as template DNA in the sequence reactions, the nucleotide sequence was determined for a fragment of 1.6 kbp, located upstream of the above-described HindIII-BcII fragment on the chromosome of A. calcoaceticus BD413. This
completed the sequence of a 3,669-bp fragment encompassing the A. calcoaceticus lipase-encoding region (Fig. 4).

Analysis of the lipB upstream sequence revealed one complete ORF, designated lipB, that had been disrupted in AAC 302. This ORF is transcribed in the same direction as lipA and is located 135 nucleotides upstream of the lipase-encoding gene (Fig. 4). The translation initiation codon of the lipB ORF most likely is the ATG-601 codon, preceded by a putative ribosome binding site (GGA-588), similar to the ribosome binding site upstream of the lipA gene (43), yet with a relatively long spacing of 10 nucleotides. This sequence has a calculated free energy for binding to the 3' end of the 16S rRNA of A. calcoaceticus (5' -GATACCTTCCTT-3' [75a]) of 30.2 kJ/mol. The translation stop codon TAA-1630 delineates the 3' end of the lipB ORF (Fig. 4).

The lipB gene encodes a protein of 343 amino acids, LipB, with a predicted molecular mass of 39.0 kDa. Interestingly, A. calcoaceticus LipB is highly similar to chaperone-like proteins that have been found to be indispensable in the production of extracellular lipase in several species of Pseudomonas (Table 3). A. calcoaceticus LipB is especially similar along its complete amino acid sequence to the LipB chaperones of P. aeruginosa TE3285 (7) and P. aeruginosa PA01 (76) and to LimL of Pseudomonas sp. 109 (31) (Table 3 and Fig. 5). Comparison of the corresponding lipase primary structures revealed identical strain groupings (12, 18, 43): the lipases of the above organisms, including LipA of A. calcoaceticus BD413 (43), form a distinct subgroup, separate from the lipases produced by Pseudomonas glumae PG-1 (15), P. cepacia (35), and Pseudomonas sp. KWI-56 (32).

Upstream of the lipB ORF, part of an additional ORF, orfX, which is divergently transcribed, was identified (Fig. 4). Translation of orfX presumably is initiated at position 455 (ATG-455), only 145 bp upstream of the lipB ORF. A weak ribosome binding site for orfX may be formed by AGA-64 (Fig. 4B). The 152-amino-acid translation product of orfX shows high similarity to the hypothetical P35 protein of E. coli (64) (61.3% identical and 32.3% similar residues in 142 amino acids that overlap). The 25.6% similar residues in 64 amino acids that overlap) and also to a (partial) hypothetical protein encoded by orf7 in the infB region of Bacillus subtilis (67a) (database accession no. P32732; 50.0% identical and 25.6% similar residues in 64 amino acids that overlap). The function of the protein encoded by A. calcoaceticus orfX is as yet unknown. Whether the predicted orfX-encoded protein is involved in lipase production is presently under investigation.

Analysis of a LipB-PhoA fusion peptide. Frenken et al. (13) have determined that the LipB protein of P. glumae is N terminally anchored in the cytoplasmic membrane, while the largest (C-terminal) part of the protein is located in the periplasm, where it asserts its function, presumably in folding of the lipase (14). Like the Pseudomonas lipase chaperones, the putative LipB protein of A. calcoaceticus BD413 contains a potential N-terminal membrane-spanning helix, from residues Leu-9 to Met-25 (Fig. 4A), as determined by the program SOAP (PC/Gen), suggesting a similar localization and orientation for the Acinetobacter protein. To determine whether the C-terminal part of the A. calcoaceticus LipB protein is located outside the cytoplasm, a translational lipB:phoA fusion was generated at the HindIII site in the lipB gene, by use of the phoA cassette of plasmid pPHO7 (20) (Fig. 6). Plasmid pALJA26, carrying this lipB:phoA fusion, was allowed to integrate into the chromosome of A. calcoaceticus BD413, via homologous recombination at sequences in or upstream of lipB. The resulting Acinetobacter strain, AAC324, clearly produced alkaline phosphatase activity, whereas BD413 did not show any such activity. Plasmid pALJA26 also conferred alkaline phosphatase activity, whereas BD413 did not show any such activity. Plasmid pALJA26 also conferred alkaline phosphatase activity.

**FIG. 4.** (A) Nucleotide sequence of a chromosomal fragment of 1,800 bp, encompassing the complete lipase chaperone gene lipB and part of orfX and amino acid sequence of the predicted lipase chaperone LipB. The sequence of nucleotides 1611 to 1800, carrying the start of the lipase-encoding gene lipA, has also been reported elsewhere (43). (B) Inverted nucleotide sequence of residues 525 to 1, including the predicted amino acid sequence of OrfX. Nucleotide residues are numbered to the right (in boldface), together with the predicted amino acids (in italics) with the predicted protein sequences as follows: (75) **A. CALCOACETICUS**
line phosphatase activity on E. coli DH5α, whereas pPHO7 alone was unable to do so (Fig. 6). Since alkaline phosphatase is enzymatically active only when it is transported out of the cytoplasm, the results obtained with the LipB-PhoA fusion protein indicate that the C-terminal part of the LipB protein of A. calcotabicus chaperone is indeed located outside the cytoplasm, even in the heterologous host E. coli. Together, this suggests that also in A. calcotabicus the LipB protein is (partly) located in the periplasm and, as has been found in Pseudomonas spp., N. gonorrhoeae, and H. influenzae, it is also transiently anchored to the cytoplasmic membrane.

lipoB and lipA form a bicistronic operon. To investigate whether A. calcotabicus BD413 lipB and lipA are independently expressed, or whether they in fact form a transcriptional unit, the following two in trans complementation experiments were performed: (i) complementation of a lipA mutant with a plasmid carrying only lipA and (ii) complementation of lipB disruption strain AAC302 with a plasmid carrying lipB, but not lipA. The lipA gene was subcloned on a 2.1-kbp EcoRI-SalI fragment of mpALJA2 into Agrobacterium E. coli shuttle vector pWH1274, to allow for its replication in Agrobacterium spp. (see Materials and Methods). The resulting plasmid, pALJA230 (Fig. 3), was used to complement A. calcotabicus lipA mutant AAC302-1. This strain was chosen since it carries a marker inserted in the chromosomal copy of lipA (42), which provided the possibility of screening for the presence of the insertion mutation. To prevent high-frequency integration of pALJA230 into the chromosome of the recipient strain, via homologous recombination, the recA gene in AAC320-1 was first disrupted (resulting in AAC320-1rec; see Materials and Methods). After transformation of pALJA230 into AAC320-1rec, transformants were screened for the production of lipase on egg yolk indicator plates. No complementation of the lipase-negative phenotype was observed. This indicates that the lipA gene indeed is not expressed on pALJA230, even though the plasmid carries 0.5 kbp of wild-type DNA upstream of lipA (Fig. 3 and 4). Apparently, the lipA expression signals are not encoded in this upstream region, suggesting that regulation of lipA expression is brought about from sequences even further upstream of this region on the chromosome of A. calcotabicus.

We also tested whether mutant AAC302 could be complemented by plasmid pALJA25-1 (Fig. 3). This plasmid carries lipB (apart from five of its 3' codons) on a 5.8-kbp HindIII fragment, derived from the insert in pAVA302-1 (see Materials and Methods). Also in this complementation experiment, the chances of integration of the vector into the chromosome of the recipient were reduced by inactivation of recA (see Materials and Methods). Like AAC320-1rec(pALJA230), the resulting strain, AAC320-1rec, carrying plasmid pALJA25-1, also failed to produce lipase activity. Via restriction analysis of plasmid pAKA302 (Fig. 3), it could be shown that on the AAC302 chromosome, the nptII gene is located directly upstream of the BclI restriction site at position 928 in the lipB ORF, thereby partly disrupting the gene encoding the lipase chaperone (Fig. 4). Therefore, expression of the lipA gene should be mediated upstream of this site, which is located only 327 bp from the 5' start of the lipB ORF. The mutation in AAC302 could be complemented by chromosomal insertion of the nonreplicating plasmid pALJA432 into the chromosome of AAC302. This indicates that the lack of production of lipase in AAC302 is not caused by secondary factors, since pALJA432 only carries the complete lipBA gene cluster (Fig. 3) (43).

These results strongly suggest that transcription of lipA is indeed initiated upstream of the gene encoding the lipase chaperone. Identification of the divergently oriented orfX, upstream of lipB, indicates that lipB and lipA in fact form a bicistronic operon. In addition, AAC302 can be classified as a mutant of A. calcotabicus BD413, in which the expression of the complete lipBA operon is disrupted, consistent with the LipA- phenotype of this strain (Table 2 and Fig. 2).

Primer extension analyses: identification of a putative lipBA promoter. To investigate the presence of a potential transcription initiation site(s) upstream of lipB, primer extension analyses were performed, by using a lipB-internal primer (LipB1 [Fig. 4A]), on mRNA preparations of early-stationary-phase cultures of A. calcotabicus BD413 grown in N broth. As control strains for primer extension experiments, strains BD413 (pALJA25-1) and BD413(pAVA302-1) were used. In BD413 mRNA, only one (major) +1 signal was detected, 51 bases upstream of the translation initiation codon of the lipB ORF (Fig. 7), indicating that the corresponding nucleotide, C-550, is the nucleotide which is primarily used as the initiation site for lipB transcription. Several other bases may serve as transcription initiation sites as well (A-547, T-549, and A-551 [Fig. 7 and 4A]), but these sites apparently are less frequently used. Comparison of the wild-type strain (BD413, lane 1 in Fig. 7) with the two strains carrying the lipA gene in multiple copies [BD413(pALJA25-1), lane 2, and BD413(pAVA302-1), lane 3] shows that much higher levels of the lipB transcript are produced in the multicopy strains: primer extension signals in these mutants are much stronger than in the wild type. Though the minor signals are also clearly stronger, the multicopy primer extension signals identify C-550 as the primary +1

### Table 3. Similarity of Pseudomonas lipase chaperones to A. calcoaceticus BD413 LipB

<table>
<thead>
<tr>
<th>Lipase chaperone similar to LipB of A. calcoaceticus BD413 (organism)</th>
<th>% of amino acids identical to LipB</th>
<th>% of amino acids similar to LipB</th>
<th>Reference (no. of overlapping amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LipB (P. aeruginosa TE3285)</td>
<td>30.2 + 47.2 (341)</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>LimB (Pseudomonas sp. 109)</td>
<td>30.7 + 46.5 (342)</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>LimA (P. cepacia DSM5959)</td>
<td>22.9 + 50.9 (328)</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>LimL (Pseudomonas sp. KWI-56)</td>
<td>22.3 + 49.0 (328)</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>LipB (P. glomeris FG1)</td>
<td>24.3 + 47.9 (317)</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

* Sequences were compared according to the method of Pearson and Lipman (55) with the FastA program.
nucleotide as well (Fig. 7). Also in this case, longer fragments were not detected in the primer extension analysis, suggesting that C-550 does not simply represent a preferred fall-off site of reverse transcriptase, on a transcript that is initiated further upstream of lipB. The additional signals in lanes 2 and 3, below the proposed transcription initiation signals, most likely reflect transcript degradation or fall-off products of reverse transcriptase.

A potential promoter, similar to the *E. coli* σ^70^ consensus (25, 28, 62), was identified upstream of the nucleotide shown to be the major transcription initiation site. A putative −35 region begins at T-513 (TTGTAT) and may act in combination with a −10 region which begins at T-538 (TATTTT; essential bases are underlined [24]). These two regions are separated by 19 nucleotides (Fig. 4A), which is relatively far apart compared with the optimal spacing in *E. coli* (17 bp [24]). Interestingly, a 19-bp spacing has also been found for the trpFB operon of *A. calcoaceticus* BD413 (23). The −35 region of the putative lipB promoter is also identical to the equivalent region upstream of the *A. calcoaceticus* trpE gene. However, this region is separated from its consensus −10 region (TATAAT) by only 16 nucleotides (23).

Identification of the divergently transcribed orfX' sequence, only 145 bp upstream of the lipB gene, may have consequences for the regulation of expression of the lipBA operon. As has been indicated above, translation of orfX' presumably is initiated from the ATG codon at position 455 (Fig. 4B). No primer extension analysis has been performed for orfX', and yet no clear consensus promoter structure could be derived from the sequence upstream of orfX'. However, it is quite possible that the regulatory sequences of the lipBA operon (partly) overlap with those of orfX', in which case initiation of transcription of each of the two operons may be influenced by the other.

**LipB is essential for lipase production.** The identification of the lipB gene strongly suggests that the encoded LipB protein, as in *Pseudomonas* spp., is required for the production of active extracellular LipA. However, since transcription of lipA has also been abolished by the polar mutation in the lipB gene in AAC302, the function of LipB as an essential protein in lipase production in *A. calcoaceticus* BD413 remains to be established. This was investigated by the following complementation experiment. Lipase gene lipA was cloned into the tetracycline resistance gene of plasmid pWH1274, such that lipA transcription would be directed from the tet promoter. The plasmid carrying this construct, pALJA26, subsequently was transformed into *A. calcoaceticus* AAC302 rec and AAC320-1 rec (see above), and transformants were screened for lipase production on egg yolk plates. AAC320-1 rec (pALJA27) did form a turbid zone around its colonies, indicating that LipA is produced in this organism and that lipA is indeed expressed from pALJA27. However, AAC302 rec (pALJA27) failed to produce lipase activity. This shows that the presence of LipB is indeed essential for the production of lipase in *A. calcoaceticus* BD413.

**DISCUSSION**

Production of so-called group I extracellular lipases in *Pseudomonas* spp. requires the involvement of a lipase-specific helper protein that is anchored in the cytoplasmic membrane...
with a large C-terminal domain in the periplasm (for reviews, see the work of Gilbert [18] and Jaeger et al. [34]). The exact function of such helper proteins has yet to be fully elucidated. Strong evidence has been presented on their role in mediating conformational changes in the inactive lipase protein during its passage through the periplasm, designating them as chaperone-like proteins (14, 27). Identification and characterization of the lipB gene of A. calcoaceticus BD413 indicate that a lipase-specific chaperone is also active in this organism. As in Pseudomonas spp., Acinetobacter LipB is essential for LipA production, since lipA expression plasmid pALJA27 fails to complement the lipase-negative phenotype of lipBA mutant AAC302rec. Analysis of the amino acid sequence of LipB suggests that LipB in A. calcoaceticus is N terminally anchored to the cytoplasmic membrane. Moreover, as shown by analysis of LipB-PhoA fusion constructs, the C-terminal part of LipB is located outside the cytoplasm, presumably in the periplasm, identical to the situation in Pseudomonas spp. This lends further support to the proposed model, which suggests that the mechanisms of lipase production in A. calcoaceticus and in several pseudomonads are highly similar (see below). Furthermore, identification of a lipase chaperone in A. calcoaceticus suggests that similar proteins may also be present in other gram-negative lipase-producing bacteria.

Despite many similarities, a striking difference between A. calcoaceticus BD413 and several pseudomonads has been revealed with respect to the genomic organization of the genes encoding the lipase and its specific chaperone. Acinetobacter lipB is located upstream of the structural gene for the lipase, in contrast to the situation in Pseudomonas spp., in which genes encoding lipase-specific chaperones have invariably been localized downstream of their respective structural lipase genes (34). Frenken et al. (13) have presented evidence that the two functionally coupled genes in P. glumae are also transcriptionally coupled. Interestingly, results from primer extension and complementation analyses indicate that Acinetobacter lipB and lipA are transcriptionally coupled as well and also form a bicistronic operon. These findings strongly suggest that the function of the helper protein both in A. calcoaceticus and in Pseudomonas spp. is primarily, if not solely, dedicated to the lipase.

Identification of the A. calcoaceticus lipB gene upstream of the structural lipase gene lipA has several important regulatory implications. During growth of A. calcoaceticus BD413 in batch cultures, depending on the medium used, formation of both the extracellular lipase LipA and the intracellular esterase EstA is specifically induced when the culture reaches the stationary phase (40, 43). The growth phase dependence ofEstA production has been shown to be controlled at the transcriptional level (40). Also, the lipA gene may be regulated in a growth phase-dependent manner. Identification of the lipase gene as part of a bicistronic operon together with the upstream-located lipB gene indicates coordinate regulation of the lipB and lipA expression. Identification of a putative ρ5-like promoter upstream of lipB suggests that expression of the lipBA operon is controlled by the main vegetative σ factor of A. calcoaceticus BD413. Growth phase-dependent lipBA expression will therefore presumably require additional regulatory factors. This is currently being investigated.

The stoichiometry of expression of the two proteins, LipB and LipA, which is of importance in view of the biological function of the helper protein in lipase production, has remained a point of debate. Recent publications have presented conflicting evidence on this subject. Hobson et al. (27) have shown association of the helper protein (Lim) with the lipase protein (LipA) of P. cepacia and have reported Lim (designated a lipase chaperone) to be essential for in vitro refolding of LipA into an active conformation, after denaturation of LipA with urea. Their experiments suggested a 1:1 stoichiometry of production of these two proteins (27). In contrast, evidence presented by Frenken et al. (14) suggests that the lipase helper protein (LipB) of P. glumae is not required for activation of the lipase (LipA). After unfolding LipA in guanidine-HCl, these authors were able to obtain refolding of the lipase into an active state in the absence of the LipB protein. They propose a catalytic function for LipB in the folding of the lipase into an export-competent state (the LipB protein is therefore designated a lipase foldase by these investigators), and hence a 1:1 molar ratio of the production of the two proteins is not required (14). Potential differences in production of LipA and LipB in P. glumae have been hypothesized to be caused by

**FIG. 7.** Autoradiogram showing part of the nucleotide sequence upstream of lipB (lanes T, G, C, and A), together with the primer extension signals (lanes 1, 2, and 3), obtained with radiolabelled oligonucleotide LipB1 (see Fig. 4A) as primer. The bases corresponding to transcription initiation sites of the lipB transcripts are indicated by arrows and shown in boldface in the derived nucleotide sequence. Long arrow, major signal (+1: also indicated by an arrow in the primer extension lanes); short arrows, minor signals (see text). A base shift of the primer extension signal of roughly 1.5 bases relative to the sequence signals has been taken into account, since in the primer extension reactions the primer was labelled (5'-[32P]P), whereas sequence reactions were performed with [α-32P]dATP and unlabelled ro3 primer (68). Lane 1, BD413; lane 2, BD413 (pALJA25); lane 3, BD413(pAVA302-1).
differential (in)stability of the lipAB transcript and by partial termination of lipA transcription upstream of lipB, since the lipAB-intergenic region has an extremely high G+C content and carries several inverted repeats (13). However, since in A. calcoaceticus BD413 the order of the two genes is reversed, a similar hypothesis is not tenable for this organism. Apart from potential differences in translation initiation frequency or half-life between the two proteins, it is expected that regulation of their production at the genetic level does not allow for large differences in the stoichiometry of their expression. Actually, the inverse order of the two genes in A. calcoaceticus BD413, compared with Pseudomonas spp., together with their coregulated expression in both organisms, may suggest that the two proteins are produced in equimolar amounts and argues against a purely catalytic function of the periplasmic lipase chaperone.

Though the helper protein may induce the formation of certain secondary structure elements in the lipase protein, essential for translocation of the lipase across the outer membrane (14), it may especially be of high importance in preventing the lipase from folding in an active conformation in the periplasm before it has been translocated across the outer membrane. In this case, a stoichiometric production of LipB and LipA, such as also found by Hobson et al. (27), could be essential. In summary, this suggests a true chaperone function for the lipase-specific helper protein in Acinetobacter and Pseudomonas species, reminiscent of the cytoplasmic molecular chaperone SecB in E. coli. Apart from mediating folding of certain cytoplasmic proteins, SecB also prevents folding of exoprotein precursors that must be translocated across the cytoplasmic membrane via the Sec secretion system and prevents formation of insoluble protein aggregates (17, 52, 59). In addition, the association between the lipase and its chaperone may prevent degradation of the lipase by proteases, such as found by Frenken et al. (14). Prevention of the lipase from folding into an active conformation may also protect the host organism from potentially harmful degradation of cellular lipids in the periplasm.

In the current model for lipase export in A. calcoaceticus (Fig. 8), the immature lipase, carrying an N-terminal signal sequence of 20 amino acids, is transported across the cytoplasmic membrane via a Sec-like protein translocation system (17, 59), with simultaneous cleavage of the leader peptide by a periplasmic signal peptidase (17, 59, 72). The processed lipase protein (303 amino acids) subsequently is translocated across the outer membrane and exported to the extracellular medium, via a separate protein translocation complex. Such a system, which has been termed Xcp in Pseudomonas spp. (3, 4, 50), was first detected in Klebsiella spp. (Pul [60]) and probably recognizes structural elements in the peptides to be translocated (69). We have previously reported that the periplasmic peptidyl prolyl cis-trans isomerase (rotamase or RotA) of A. calcoaceticus BD413 is not required for lipase production (41). However, a periplasmic protein, disulfide oxidoreductase (PdoA), does play a role. Such periplasmic processing enzymes may also be involved in lipase production in Pseudomonas spp., since group I and II Pseudomonas lipases have been shown to contain a disulfide bridge (34). In the periplasm, the protein disulfide oxidoreductase in A. calcoaceticus presumably facilitates disulfide bond formation between the only two cysteine residues in the LipA polypeptide, which is thought to be essential for further translocation across the outer membrane (43, 58, 69). Thus in this model, the periplasmic lipase protein is expected to contain at least some secondary structure before it is translocated across the outer membrane by the Xcp system. However, as is explained above, we hypothesize that formation of extensive secondary-tertiary structure is prevented by association of the lipase with its dedicated membrane-bound periplasmic chaperone.

Four of five lipase-deficient mutants, isolated from A. calcoaceticus, have been identified as pleiotropic mutants, impaired in the production of both extracellular lipase and the Tween 80-degrading esterase. From accumulation of the periplasmic form of the LipA protein in these strains, it can be concluded that export of the lipase across the outer membrane cannot proceed. This strongly suggests that mutants AAC300, -303, -304, and -305 are affected in the Acinetobacter equivalent of the Xcp system. This is not unlikely, since xcp mutants of Pseudomonas spp. have been localized to several (large) gene clusters, and unlike sec mutants (66), xcp mutations are not lethal (69). Initial attempts to complement the Acinetobacter mutants with xcp genes of P. aeruginosa have failed. Broad-host-range plasmid pAX24, kindly provided by A. Filloux, carries nine of the xcp genes that have thus far been identified in P. aeruginosa (xcpR to +Z [3, 11]). Introduction of this plasmid into strains AAC300, -303, -304, and -305 did not result in complementation of their lipase-negative phenotype, as screened on plates containing egg yolks (44a). However, apart from the obvious possibility that additional genes may have been disrupted in the mutants, the observed lack of complementation may also be due to lack of expression of the Pseudomonas genes in A. calcoaceticus, since these organisms strongly differ in chromosomal G+C content (67.2 mol% for P. aeruginosa [51] versus 38 to 45 mol% for Acinetobacter spp. [26]). Moreover, lack of protein export in complementation experiments across species barriers has often proven difficult (69). Further experiments now aim at mapping of the mutations in AAC300, -303, -304, and -305 and complementation of these Acinetobacter mutants with chromosomal DNA of wild-type BD413.

![Model of the two-step secretion route of the lipase protein LipA of A. calcoaceticus BD413 (see text), derived from the work of Palmen et al. (52). The thin lines represent the LipA protein being transported to the periplasm by the Sec system (thin zigzag line representing the N-terminal signal peptide in the immature lipase) and translocated via the Xcp system, folded into the active conformation in the extracellular medium. The periplasmic protein disulfide oxidoreductase (PdoA) and the lipase foldase protein (LipB) have been indicated as well. Major components of the two protein translocation systems have been indicated.](image)
A large part of the above-proposed model for export of the lipase will also be applicable to export of the Tween esterase: *A. calcoaceticus* xcp mutants would fail to deliver the enzyme(s) responsible for degradation of Tween 80 to its (their) proper extracellular location, concurrent with the absence of Tween esterase activity in mutants AAC300, -303, -304, and -305. Recent experiments have indicated that the four presumed export mutants of *A. calcoaceticus*, in comparison with wild-type BD413 and lipBA mutant AAC302, are also strongly impaired in their capacity to grow on long-chain alkanes, such as hexadecane (39a). This may reflect the requirement of extracellular proteins, involved either directly in alkane degradation or indirectly via the production of bioemulsifiers during growth on hexadecane. In this respect, it is interesting that an esterase has been identified in *A. hwoffi* RAG-1 (formerly *A. calcoaceticus*), which presumably is exported and bound to the outer surface of the cell (1, 61, 67). The esterase is involved in release of the bioemulsifier emulsan (21, 67), an anionic heptacosaaccharide which is essential for the organism’s ability to grow on crude oil and other hydrocarbons (21). An emulsifying biopolymer is also produced by *A. calcoaceticus* BD413 (38, 39). *A. calcoaceticus* BD413 may produce an esterase, which, equivalent to the esterase of RAG-1, may be involved in release of the emulsifier from the cells. Translocation of such esterase activity (e.g., the Tween esterase) across the outer membrane may be impaired in mutants AAC300, -303, -304, and -305.

The phenotypic characterization of the mutant strains of *A. calcoaceticus* BD413, as described in this paper, has also led to the identification of two new lipolytic enzymes in this organism, a tributyrin-degrading esterase and an esterase responsible for degradation of Tween 80. Mutants AAC300, -303, -304, and -305 show residual tributyrin degradation, whereas they are totally devoid of Tween 80 degradation (Table 2), indicating that these two ester-hydrolyzing activities are caused by two different esterases, of which the Tween esterase is proposed to be exported across the outer membrane (see above). Interestingly, Claus et al. (9) have described the identification of an esterase in the outer membrane fraction of *A. calcoaceticus* 69/V, which is active at the outer surface of intact cells, reminiscent of the Tween esterase of BD413. The hydrolytic properties of this esterase of 69/V are also reminiscent of those of the BD413 Tween esterase. Also, the 69/V enzyme showed high specific hydrolytic activity towards Tween 80, and yet tributyrin degradation by this enzyme could not be detected (9).

*A. calcoaceticus* BD413 apparently produces multiple lipolytic enzymes, a trait which is shared with numerous other *Acinetobacter* spp. In fact, electrophoretic patterns of esterases have even been used in epidemiological typing of potentially pathogenic acinetobacters (57). Recent evidence has shown that even a fifth esterase is produced by *A. calcoaceticus* BD413. However, deletion of the gene encoding this fifth esterase does not affect the lipolytic activity of BD413 towards the above-described lipidic substrates, under any of the conditions tested so far (39a).

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