Growth-phase-dependent expression of the lipolytic system of *Acinetobacter calcoaceticus* BD413: cloning of a gene encoding one of the esterases

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Acinetobacter calcoaceticus BD413, when grown in batch culture in nutrient broth, produces both extracellular lipase activity and cell-bound esterase activity during and after the transition between exponential growth and the stationary phase. From a library of *A. calcoaceticus* DNA in *Escherichia coli*, plasmids were isolated that enabled *E. coli* to grow on media with tributyrin as the sole carbon source. Assays with model substrates classified the product of the cloned gene as an esterase. Via deletion analysis, the esterase gene was mapped on a 1·8 kbp chromosomal DNA fragment. This fragment was sequenced and found to contain one open reading frame, termed *estA*, which encodes a protein of 40·0 kDa. The amino acid sequence of this protein shows homology to a number of lipolytic enzymes, most notably to esterases. Deletion of *estA* only partially abolished cell-bound esterase activity in *A. calcoaceticus*, indicating that BD413 forms at least two esterases. Both esterases show the same temporal regulation of expression. β -Galactosidase activity was measured in strains in which a promoterless *lacZ* gene was inserted into *estA*. Induction of *lacZ* expression in these strains also occurred at the end of exponential growth in batch cultures, indicating that production of the esterase is regulated at the genetic level.

Introduction

Cessation of growth in bacterial batch cultures triggers the coordinate expression of many important processes. Amongst these are sporulation (Losick *et al.*, 1989), starvation and stringent response (Cashel & Rudd, 1987; Goldman & Jakubowski, 1990), induction of competence for natural transformation (Dubnau, 1991), the production of secondary metabolites (Hopwood *et al.*, 1986; Martín & Liras, 1989) and the production of a wide variety of enzymes (Daza *et al.*, 1991; Lange & Hengge-Aronis, 1991).

The molecular mechanisms underlying the regulation of these phenomena are gradually being elucidated. Recent reports suggest that the expression of several of these growth-phase-dependent processes is controlled through central regulatory programmes (Lange & Hengge-Aronis, 1991; Matin, 1991; Siegle & Kolter, 1992). They may involve specific nutrient limitations, uncharged tRNAs (Goldman & Jakubowski, 1990), positive and negative transcriptional regulators (Hoopes & McClure, 1987), highly selective and specific promoter structures (Stragier & Losick, 1990), alternative σ -subunits of RNA polymerase (Helmann & Chamberlin, 1988; Stragier & Losick, 1990) and post-transcriptional control of regulatory enzymes (Craig & Gross, 1991).

We are interested in the physiological and genetic factors that regulate and control the expression of growth-phase-dependent genes. As a model we selected the lipolytic system of *Acinetobacter calcoaceticus* BD413 (Juni & Janik, 1969). Several *Acinetobacter* have been shown to produce lipolytic enzymes (Breuil & Kushner, 1975; Shabtai & Gutnick, 1985; Haferburg & Kleber, 1983). However, the regulation of the production of these enzymes has not been addressed at the molecular level. Esterases and lipases are carboxylic ester hydro-

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Abbreviations: pNP, *p*-nitrophenol; pNPA, *p*-nitrophenyl acetate; pNPP, *p*-nitrophenyl palmitate; RBS, ribosome-binding site.

The GenBank accession number for the sequence reported in this paper is X71598.

lases (EC 3.1.1) that generally have an essential serine residue at their active site (Brady *et al.*, 1990). The carboxylesterases (EC 3.1.1.1) hydrolyse water-soluble or emulsified esters with relatively short fatty acid chains, whereas lipases (triacylglycerol acyl hydrolases; EC 3.1.1.3) preferentially attack emulsified substrates with long-chain fatty acids. *A. calcoaceticus* strain BD413 produces cell-bound esterase activity (Gutnick *et al.*, 1991) and extracellular lipase activity. Additionally, the state of high competence for natural transformation makes this organism, more than other *Acinetobacter*s, ideally suited for molecular studies (Juni, 1972; Palmen *et al.*, 1993).

In this report, we describe the expression of the lipolytic system of *A. calcoaceticus* BD413, as well as the cloning and characterization of one of the genes involved, encoding a cell-bound esterase.

Methods

Bacteria and plasmids. These are listed in Table 1.

Media and culture conditions. Strains of *Acinetobacter* and *E. coli* were grown in LB medium [5 g NaCl, 5 g Yeast Extract (Difco) and 10 g Bacto-Tryptone (Difco) per litre (pH 7.4)] or nutrient broth (N-broth; Gibco). The basis for the minimal medium, with tributyrin as the sole carbon and energy source, consisted of 100 mM-NH₄Cl, 10 mM-KCl, 2 mM-Na₂SO₄, 1.25 mM-MgCl₂, 20 µM-CaCl₂, 2 mM-citric acid and, per litre, 5 ml of a trace-element solution containing 5 mM-ZnO, 20 mM-FeCl₃, 10 mM-MnCl₂, 1 mM-CuCl₂, 2 mM-CoCl₂, 1 mM-H₃BO₃, 16 µM-Na₂MoO₄ and 100 mM-HCl. After sterilization, 50 mM-NaP_i buffer (pH 7.0) and 1% (v/v) tributyrin were added to the basal medium. Proline and thiamin were added to a final concentration of 100 µg ml⁻¹ and 1 µg ml⁻¹, respectively. For plates, liquid media were solidified with 1.5% (w/v) agar.

For esterase indicator plates, 3 ml of an emulsion of 50% (v/v) tributyrin and 5% (w/v) gum arabic was added to 100 ml of molten N-broth agar medium. Tributyrin was emulsified, using a Branson 250 sonifier and a microtip, for 3 min at 75 W (duty cycle 100%). Esterase production on these plates was detected by the formation of a zone of clearance around the colonies.

Two types of lipase indicator plates were used. (1) Egg-yolk plates. For these, 1.5% (v/v) egg-yolk emulsion (Oxoid) was added to molten N-broth agar containing 1% (w/v) NaCl. Lipase production on these plates gives a turbid halo around a colony (Willis & Gowland, 1962; Nitsch & Kutzner, 1969). (2) Olive oil plates. For these plates, 2% (v/v) olive oil and 0.04 mM-rhodamine B were added to molten N-broth agar, containing 1% (w/v) NaCl. Production of lipase(s) on these plates is detected by the formation of a fluorescent zone around the colonies (Kouker & Jaeger, 1987).

Antibiotics were used at the following final concentrations, in plates and in liquid media: ampicillin, 100 µg ml⁻¹; chloramphenicol, 50 µg ml⁻¹ for *A. calcoaceticus* and 20 µg ml⁻¹ for *E. coli*; kanamycin, 15 µg ml⁻¹ for *A. calcoaceticus* and 50 µg ml⁻¹ for *E. coli*; tetracycline, 15 µg ml⁻¹.

Chemicals. Restriction enzymes and T4 DNA ligase were used as recommended by the manufacturer (Pharmacia LKB Biotechnology).

DNA isolation. Chromosomal DNA was isolated as described by Vosman & Hellingwerf (1991). Plasmid DNA was purified by the method of Ish-Horowitz & Burke (1981). All additional molecular

genetic techniques were performed according to Sambrook *et al.* (1989).

Transformations. Plasmids were transformed into *E. coli* as described by Mandel & Higa (1970). Introduction of plasmid DNA into *A. calcoaceticus* BD413 by natural transformation was performed as described by Palmen *et al.* (1993).

Sequence analysis. The nucleotide sequence of fragments, cloned in M13mp18 and M13mp19, was determined from both strands by the dideoxy-chain-termination method as described by Sanger *et al.* (1977), adapted for Sequenase version 2.0 (US Biochemical Corp.) with either the M13 universal primers or synthesized primers. The sequence data were analysed using PC/Gene (release 6.5; Intelligenetics, California) and the University of Wisconsin Genetics Computer Group (UWGGC) software (GCG package, version 7.0). The nucleotide sequence of the 1829 bp *Pvu*II/*Hpa*I fragment of *A. calcoaceticus* BD413 encoding the esterase will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession number X71598.

Southern hybridization. Southern hybridization experiments, using non-radioactive digoxigenin-DNA-labelled probes, were performed as indicated by the supplier (Boehringer Mannheim).

SDS-PAGE and immunodetection of β -galactosidase on Western blots. β -Galactosidase and the EstA- β -galactosidase fusion protein in *Acinetobacter estA-lacZ* fusion strain AAC313-1 (see below) were separated by SDS-PAGE (5%, w/v, acrylamide) according to Laemmli (1970). Proteins were then blotted onto nitrocellulose according to Towbin *et al.* (1979) in a Bio-Rad Trans-Blot Semi-Dry Transfer Cell, for 1 h at 15 V (current limit 0.5 A per gel). Western blots were immunostained using polyclonal rabbit antibodies raised against β -galactosidase (kindly provided by Dr J. Tomassen) and horseradish-peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad), in combination with HRP-Color Development Reagent (Bio-Rad). Prior to incubation with antibodies, the transfer of proteins to the nitrocellulose membrane was verified by staining with 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid. Broad-range SDS-PAGE molecular mass standards, ranging from 6.5 to 200 kDa (Bio-Rad) were used to calculate the difference in molecular mass between native β -galactosidase and the EstA- β -galactosidase fusion protein.

Measurement of enzyme activities and protein concentration. Esterase and lipase production was measured in 300 ml cultures of strains grown in N-broth without antibiotics (*A. calcoaceticus*), or in N-broth supplemented with 50 mM-KP_i (pH 7.0), 1 mM-glucose and the appropriate antibiotics (*E. coli*). Under these conditions, exponential growth and an abrupt transition to the stationary phase were observed. Cultures were inoculated at an OD₅₄₀ of 0.1, with cells from an overnight culture in the same medium. Cells were grown in MultiGen fermenter vessels (New Brunswick Scientific Co.), with saturating aeration at 30 °C (*A. calcoaceticus*) or 37 °C (*E. coli*). The OD₅₄₀ was recorded as a representation of growth.

Culture samples (5 ml) were centrifuged at 3000 g for 10 min at 0 °C. Part of the supernatant was retained for determination of extracellular lipase activity. Cells were washed once in ice-cold 20 mM-HEPES buffer (pH 7.0) and resuspended in the same buffer (5 ml). A small part of the suspension was then used for the determination of protein concentration (by the method of Bradford, 1976) and (when necessary) kept on ice for subsequent determination of β -galactosidase activity. The remainder of the suspension was sonicated on ice at 75 W (duty cycle: 50%) for 3 min using a Branson 250 sonifier with a microtip. These sonicated suspensions were immediately used for the determination of cell-bound esterase activity.

Esterase assay: 36 mg *p*-nitrophenyl acetate (pNPA; Sigma) was dissolved in 1 ml methanol. Of this stock solution, 100 µl was added to 20 ml 20 mM-HEPES (pH 7.0) to yield a final pNPA concentration of

Table 1. Strains of *E. coli* and *A. calcoaceticus*, plasmids and phages used in this study

Strain/plasmid/phage	Relevant characteristics*	Marker(s)	Reference
Strains			
<i>E. coli</i> JM83	$\Delta lac(proAB)$, <i>thi</i> , ($\phi 80 lacZ\Delta M15$)		Yanisch-Perron <i>et al.</i> (1985)
<i>E. coli</i> DH5 α	$\Delta lacU169$ ($\phi 80 lacZ\Delta M15$) <i>recA1 thi-1 relA1</i>		Hanahan (1983)
<i>E. coli</i> TG1	<i>supE hsd$\Delta 5$ thi $\Delta(lac-proAB)$ F'[traD36 <i>proAB</i>⁺ <i>lacI</i>^q <i>lacZ</i>$\Delta M15$]</i>		Gibson (1984)
<i>A. calcoaceticus</i> BD413	Wild-type		Juni (1972)
<i>A. calcoaceticus</i> AAC311-2†	($\Delta 4.3$ kbp <i>estA</i> , <i>EI</i>)::(3.5 kbp <i>Cm</i> , <i>EI</i>)	<i>Cm</i> ^r	This study
<i>A. calcoaceticus</i> AAC312†	($\Delta 2.4$ kbp, <i>Hp/Sm</i>)::(3.5 kbp <i>Cm</i> , <i>EI</i>)	<i>Cm</i> ^r	This study
<i>A. calcoaceticus</i> AAC313-1†	<i>estA</i> ::(4.3 kbp <i>lacZ</i> - <i>Km</i> ($\rightarrow$), <i>S</i>), ($\Delta 0.34$ kbp <i>estA</i> , <i>S</i>)	<i>Km</i> ^r	This study
<i>A. calcoaceticus</i> AAC313-2†	<i>estA</i> ::(4.3 kbp <i>lacZ</i> - <i>Km</i> ($\leftarrow$), <i>S</i>), ($\Delta 0.34$ kbp <i>estA</i> , <i>S</i>)	<i>Km</i> ^r	This study
Plasmids and phages			
M13mp18	<i>alacZ</i>		Yanisch-Perron <i>et al.</i> (1985)
M13mp19	<i>alacZ</i>		Yanisch-Perron <i>et al.</i> (1985)
pAKA10	pUN121, <i>Bc</i> :: <i>estA</i> (5.4 kbp, <i>Sau</i>)	<i>Ap</i> ^r , <i>Tc</i> ^r	This study
pAKA20	pUN121, <i>Bc</i> :: <i>estA</i> (5.7 kbp, <i>Sau</i>)	<i>Ap</i> ^r , <i>Tc</i> ^r	This study
pAKA24	pUN121, <i>Bc</i> :: <i>estA</i> (5.3 kbp, <i>Sau</i>)	<i>Ap</i> ^r , <i>Tc</i> ^r	This study
pAKA24-5	pUN121, <i>Sm/EI</i> :: <i>estA</i> (2.8 kbp, <i>Pv/EI</i>)	<i>Ap</i> ^r , <i>Tc</i> ^r	This study
pAKA10-1†	(7.4 kbp pAKA10, <i>Hp/Sm</i>)::(3.5 kbp <i>Cm</i> , <i>Sm</i> , of pMTL24p- <i>Cm</i>) ($\Delta 2.4$ kbp, <i>Hp/Sm</i>)	<i>Ap</i> ^r , <i>Cm</i> ^r , <i>Tc</i> ^r	This study
pAKA1024-3†	(2.2 kbp pAKA10, <i>EI/Sm</i>)::(5.6 kbp pAKA24, <i>EI/EV</i>), ($\Delta 4.3$ kbp <i>estA</i> , <i>EI</i>)	<i>Ap</i> ^r , <i>Tc</i> ^s	This study
pAKA1024-32†	pAKA1024-3::(3.5 kbp <i>Cm</i> , <i>EI</i> , of pMTL24p- <i>Cm</i>)	<i>Ap</i> ^r , <i>Cm</i> ^r	This study
pAKA20A†	pAKA20 Δ (0.34 kbp <i>estA</i> , <i>S</i>)::(4.7 kbp <i>lacZ</i> - <i>Km</i> ($\rightarrow$), <i>S</i>)	<i>Ap</i> ^r , <i>Km</i> ^r , <i>Tc</i> ^r	This study
pAKA20B†	pAKA20 Δ (0.34 kbp <i>estA</i> , <i>S</i>)::(4.7 kbp <i>lacZ</i> - <i>Km</i> ($\leftarrow$), <i>S</i>)	<i>Ap</i> ^r , <i>Km</i> ^r , <i>Tc</i> ^r	This study
pKOK6	pUC4K based; contains a 4.7 kbp <i>lacZ</i> - <i>Km</i> cassette	<i>Ap</i> ^r , <i>Cm</i> ^r , <i>Km</i> ^r	Kokotek & Lotz (1989)
pKT210	IncQ, replicates in <i>E. coli</i> and <i>A. calcoaceticus</i>	<i>Cm</i> ^r , <i>Sm</i> ^r	Bagdasarian <i>et al.</i> (1981)
pMTL24p	<i>alacZ</i>	<i>Ap</i> ^r	Chambers <i>et al.</i> (1988)
pMTL24p- <i>Cm</i>	pMTL24p::(3.5 kbp <i>Cm</i> , <i>P</i> , of pKT210)	<i>Ap</i> ^r , <i>Cm</i> ^r	This study
pUN121	ColEI, <i>Tc</i> (cl)	<i>Ap</i> ^r , <i>Tc</i> ^s	Nilsson <i>et al.</i> (1983)

* The sizes (in kbp) of relevant DNA fragments and restriction enzymes used for their isolation are indicated. Restriction enzymes indicated: *Bc*, *Bcl*I; *EI*, *Eco*RI; *EV*, *Eco*RV; *Hp*, *Hpa*I; *P*, *Pst*I; *Pv*, *Pvu*II; *Sm*, *Sma*I; *S*, *Sal*I; *Sau*, *Sau*3A. *Cm*, fragment encoding chloramphenicol resistance, originally isolated from pKT210; *lacZ*-*Km*, the cassette of pKOK6 containing a promoterless *lacZ* gene and the *npII* gene encoding resistance to kanamycin. *Ap*^r, *Cm*^r, *Km*^r, *Sm*^r and *Tc*^r refer to resistance to ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline, respectively.

† The construction of plasmids and strains of *A. calcoaceticus* carrying different deletions and insertions is described in Methods.

1 mM in the assay. Sonicated cell suspension (20–200 μ l) was added to the substrate in a final volume of 2 ml. The reactions were carried out at 30 °C in a Beckman DU-40 spectrophotometer. The esterase activity was calculated from the initial rate of *p*-nitrophenol (pNP) formation, measured spectrophotometrically at 410 nm. The molar absorption coefficient of pNP at pH 7.0 was determined to be 10400 M⁻¹. One unit of enzyme activity is defined as the amount of enzyme forming 1 μ mol pNP min⁻¹.

Lipase assay: 37.5 mg *p*-nitrophenyl palmitate (pNPP; Sigma) was dissolved in 2 ml 2-propanol at 60 °C. Fifty millilitres of a buffer containing 50 mM-Tris/HCl (pH 8.0) and 0.1 % Triton X-100 was pre-warmed to 60 °C. The pNPP solution was suspended dropwise in this Tris buffer, while stirring vigorously, yielding a final pNPP concentration of 2 mM. Culture supernatant (20–200 μ l) was added to the substrate in a final volume of 2.0 ml. The reactions and activity calculations were carried out as described for the esterase assay. The molar absorption coefficient of pNP in the assay buffer (pH 8.0) was determined to be 15100 M⁻¹.

β -Galactosidase assay: the β -galactosidase activity was determined in washed cell suspensions according to Miller (1982).

Construction of mutant strains of *A. calcoaceticus* BD413. Complete and partial deletions of the esterase encoding region in the chromosome of *Acinetobacter* BD413, were generated as follows.

(1) The 0.34 kbp *Sal*I fragment of the esterase-encoding region was deleted from pAKA20 (see Results and Discussion, Fig. 2) and replaced by the *lacZ*-*Km* cassette of pKOK6, isolated as a 4.7 kbp *Sal*I fragment. This cassette contains a promoterless *lacZ* gene and the *npII* gene, encoding resistance to kanamycin (Kokotek & Lotz, 1989). The resulting plasmids, pAKA20A and pAKA20B, containing this cassette in the two orientations, were introduced into *A. calcoaceticus* BD413 via natural transformation. Prior to transformation, the plasmids were digested with *Kpn*I and *Bgl*II, both cutting in the vector part of the plasmid, to prevent replication and Campbell-like integration into the chromosome. Several kanamycin-resistant strains were isolated from transformations with both plasmids. In all strains the *Sal*I fragment of the esterase-encoding region was replaced by the *lacZ*-*Km* cassette. One of the transformants, carrying the deletion of pAKA20A, yielded strain AAC313-1; pAKA20B gave strain AAC313-2.

(2) A deletion of the complete esterase encoding region in *A. calcoaceticus* was constructed as follows. A 5.6 kbp *Eco*RI/*Eco*RV fragment of pAKA24 (left-hand fragment in Fig. 2), was ligated to a 2.2 kbp *Eco*RI/*Sma*I fragment of pAKA10 (right-hand fragment in Fig. 2). The latter fragment was isolated from a complete *Eco*RI/partial *Sma*I digestion of pAKA10. This indirectly deleted the 4.3 kbp *Eco*RI fragment present in pAKA20, containing the entire esterase-encoding region and simultaneously deleted the 890 bp *Sma*I/*Eco*RV fragment of the vector, carrying part of the tetracycline-resistance gene. The

resulting plasmid (pAKA1024-3) contained the chromosomal fragment of pAKA10 flanking the right-hand *EcoRI* site on the insert in pAKA20, and the region of pAKA24 flanking the left-hand *EcoRI* site on the insert of pAKA20 (see Fig. 2). The chloramphenicol-resistance gene of pKT210, isolated as a *PstI* fragment and cloned in the *PstI* site of pMTL24p (yielding pMTL24p-Cm), was subsequently isolated as a 3.5 kbp *EcoRI* fragment. This fragment was inserted into the unique *EcoRI* site of pAKA1024-3, between the two *EcoRI*-flanking sequences. This yielded plasmid pAKA1024-32, in which the fragment encoding resistance to chloramphenicol (Cm^r) replaced the entire 4.3 kbp *EcoRI* region encoding the esterase. pAKA1024-32 was then introduced into *A. calcoaceticus* BD413. ColE1 replicons do replicate, although poorly, in *Acinetobacter* (Vosman *et al.*, 1991). Therefore, kanamycin-resistant colonies were replica-plated onto plates containing ampicillin (resistance to ampicillin was encoded by the vector part of pAKA1024-32). Strains sensitive to this antibiotic were isolated as true deletion mutants, having replaced their esterase-encoding region by the Cm^r gene via replacement recombination. One of the resulting deletion mutants was named AAC311-2.

(3) A third type of deletion mutant of BD413, lacking the 2.4 kbp *HpaI/SmaI* fragment of pAKA10, downstream of the esterase-encoding region, was generated as follows. A plasmid (pAKA10-1) was constructed in which this fragment was replaced by the chloramphenicol-resistance gene of pKT210, isolated as a partial *SmaI* fragment of pMTL24p-Cm. To prevent replication of the plasmid, and/or its insertion into the chromosome via a Campbell-like integration, pAKA10-1 was linearized with *NruI* (which cuts in the vector part) before introduction into *A. calcoaceticus* BD413 via natural transformation. One chloramphenicol-resistant strain, carrying the deletion of pAKA10-1, was named AAC312.

Verification of chromosomal mutations via Southern hybridization.

(1) *AAC313-1* and *AAC313-2*. The 0.34 *SalI* *estA* fragment hybridizes to *SalI*-digested chromosomal DNA of wild-type BD413. However, no hybridizing signal could be found with the *lacZ*-Km insertion mutants *AAC313-1* and *AAC313-2*, showing that the *SalI* fragment was indeed deleted from these two mutants. The *lacZ*-Km cassette of pKOK6 carries two *EcoRI* restriction sites, one at the start of the *lacZ* gene, the other just at the end of the *lacZ* gene (see also Fig. 2). In *AAC313-1* one of these *EcoRI* sites is situated just downstream of the *SalI* site in *estA*; in *AAC313-2* an *EcoRI* site is introduced 1.6 kbp downstream of the *estA* *SalI* site. As expected, when the 2.0 kbp *EcoRI/SalI estA* fragment of pAKA24 was used as a probe, a 2.0 kbp hybridizing fragment was found in *EcoRI*-digested DNA of *AAC313-1* and an *EcoRI* fragment of 3.6 kbp was found to hybridize in *AAC313-2* DNA. In *EcoRI*-digested DNA of BD413, hybridization was found with the 4.3 kbp wild-type fragment.

(2) *AAC311-2* and *AAC312*. Using the 1.47 kbp *PvuII/SphI* fragment of the esterase region (see Results and Discussion, Fig. 4) as a probe, again the 4.3 kbp hybridizing fragment was found in chromosomal DNA of wild-type BD413 digested with *EcoRI*. However, this hybridizing band was absent in *EcoRI*-digested DNA of *AAC311-2*. When the 3.5 kbp *EcoRI* Cm^r fragment was used as a probe, an *EcoRI* fragment of the same size was found to hybridize in *EcoRI* digested chromosomal DNA of *AAC311-2*, whereas no hybridization was obtained with BD413 DNA. With both the *estA* probe and the Cm^r probe, a hybridizing fragment of approximately 8.5 kbp was found in *EcoRI*-digested DNA of mutant *AAC312*, confirming that the *estA* gene was still present in this mutant and that the *HpaI/SmaI* fragment had been replaced by the Cm^r fragment. As expected, neither *AAC311-2* nor *AAC312* (testing *EcoRI*-digested DNA from both strains) showed a hybridizing signal when the 1.6 kbp *HpaI/EcoRI* fragment of pAKA10 (the fragment downstream of *estA*) was used as a probe, confirming deletion of the 4.3 kbp *EcoRI* fragment in *AAC311-2* and deletion of the 2.4 kbp *HpaI/SmaI* fragment in *AAC312*.

Results and Discussion

The lipolytic system of *A. calcoaceticus* BD413

A. calcoaceticus BD413, like the psychrophilic *Acinetobacter* O16 (Breuil & Kushner, 1975), produces both esterase activity and lipase activity during growth on complex media such as N-broth (Fig. 1). The esterase activity, measured via hydrolysis of *p*-nitrophenyl acetate (pNPA), was cell-bound and remained so during prolonged incubation (not shown). In contrast, Breuil & Kushner (1975) reported that the *Acinetobacter* O16 esterase is cell-bound only during early growth and is detected in the extracellular medium late in growth. A similar situation was described for *A. calcoaceticus* RAG-1 (Shabtai & Gutnick, 1985). As has been described for *Acinetobacter lwoffii* O16 (Breuil & Kushner, 1975) and *A. calcoaceticus* 69 V (Haferburg & Kleber, 1983), lipolytic activity in cultures of BD413, measured with *p*-nitrophenyl palmitate (pNPP), was secreted into the culture supernatant. This activity could also be detected titrimetrically, using olive oil as a substrate.

In cultures of *A. calcoaceticus* BD413 grown in N-broth, 99% of the pNPA-hydrolysing activity was cell-bound, whereas roughly 96% of the pNPP-hydrolysing activity could be detected in the culture medium. We therefore conclude that the cell-bound activity is esterase activity and that a lipase is secreted into the medium. This lipase has been purified to homogeneity and characterized biochemically (R. G. Kok and others, unpublished experiments).

During growth in batch cultures in N-broth, *A. calcoaceticus* BD413 produced high amounts of esterase activity specifically during the transition between exponential growth and the stationary phase (Fig. 1). Within

Fig. 1. Production of cell-bound esterase and extracellular lipase by *A. calcoaceticus* BD413. Cultures were grown in N-broth in fermenter vessels (see Methods). ●, Growth (OD₅₄₀); ▽, specific extracellular lipase activity, measured in the culture medium with pNPP as the substrate; ▲, specific cell-bound esterase activity (NB values were 1/10 those plotted), measured in sonicated cell suspensions, with pNPA as the substrate.

Fig. 2. Schematic representation of the *A. calcoaceticus* chromosomal inserts in different plasmids and the production of esterase by strains of *E. coli* JM83 carrying these plasmids. After a few days of incubation on plates with tributyrin, esterase activity was scored by the relative size of a zone of clearance around a colony. pAKA10, pAKA20 and pAKA24 were independently isolated from the gene library in *E. coli* JM83; pAKA24-5, pAKA10-1, pAKA1024-32 and pAKA20A are derivatives of these plasmids. The insert DNA is shown as a solid thick line. Vector DNA is indicated as open boxes. Cm^r (hatched box) is the 3.5 kbp chloramphenicol-resistance gene of pMTL24p::Cm (Table 1), inserted as an *EcoRI* fragment in pAKA1024-32 and as a *SmaI* fragment in pAKA10-1. *lacZ* and Km^r (hatched arrows) refer to the cassette of pKOK6 (Table 1), inserted in two orientations between the *SalI* sites in the esterase-encoding region in pAKA20A and pAKA20B (only pAKA20A is shown). The chromosomal inserts in the plasmids are aligned. The common fragment in the different inserts, necessary for maximal esterase expression in *E. coli* JM83 is marked with dotted lines. Only the chromosomal inserts are drawn to scale. Restriction sites; *Bg*, *BglII*; *EI*, *EcoRI*; *EV*, *EcoRV*; *Hp*, *HpaI*; *Pv*, *PvuII*; *S*, *SalI*; *Sm*, *SmaI*. Restriction sites that have been lost during constructions are shown in parentheses.

90 min after growth had ceased, the specific esterase activity increased about fourfold, followed by a slower increase in activity during the next 3.5 h. From this point onwards, up to at least 19 h after exponential growth was completed, the specific esterase activity in sonicated cell suspensions remained constant (Fig. 1). This is in sharp contrast to the situation described for *A. calcoaceticus* RAG-1, which produces both cell-bound and extracellular esterase activity early during exponential growth in an ethanol/minimal salts medium. Cell-bound esterase activity in this strain decreases during further growth, and an increasing amount of esterase is detected extracellularly (Shabtai & Gutnick, 1985). Breuil & Kushner (1975) described a situation for *Acinetobacter* O16 similar to *A. calcoaceticus* RAG-1 with respect to the growth-phase dependence of esterase production. Grown on complex media, *Acinetobacter* O16 produces cell-bound esterase activity specifically during the exponential phase. Decreasing esterase activities are detected when the culture reaches the stationary phase.

During the transition period, late in the growth of *A. calcoaceticus* BD413 in N-broth, lipase activity appeared in the extracellular medium (Fig. 1). Extracellular lipase activity increased about fivefold within 60 min of the cessation of growth. Fisher *et al.* (1987) reported that the secretion of extracellular lipase of *A. calcoaceticus* 69 V is growth-phase dependent. Only the extracellular form

of the enzyme would appear at the end of exponential growth. Similar to the production of esterase, *Acinetobacter* O16 produces lipase specifically during exponential growth (Breuil & Kushner, 1975). Unlike esterase activity, the lipase activity in cultures of *A. calcoaceticus* BD413 decreased rather sharply 90 min after the stationary phase had been reached, to about the same level as was observed during exponential growth (Fig. 1). Similar lipase activity levels were detected in the extracellular medium of overnight cultures (not shown). The decrease in activity was caused by degradation of the enzyme (as could be shown with specific antibodies; data not shown).

The combination of cell-bound esterase and extracellular lipase activity, both produced at the moment of transition of a culture to the stationary phase, has not been documented thus far. Therefore, we have selected the lipolytic system of *A. calcoaceticus* BD413 as a model for our studies on the regulation of late-growth gene expression at the molecular level.

Cloning of an esterase gene of *A. calcoaceticus* BD413 via *E. coli*

When grown on N-broth plates, containing 1.5% tributyrin as an indicator of lipolytic activity (Mourey & Kilbertus, 1976), *A. calcoaceticus* BD413 formed a zone

Fig. 3. Production of esterase (a) and lipase (b) in wild-type and mutant strains of *A. calcoaceticus* BD413. Growth (OD_{540} ; ●) and specific activity of cell-bound esterase and extracellular lipase was measured in batch cultures of *A. calcoaceticus* BD413 (○), AAC311-2 (△) and AAC313-1 (▽), in N-Broth, in well-aerated fermenter vessels. One typical growth curve is shown. The production of esterase by AAC313-2 was similar to the levels produced by AAC313-1 (not shown).

of clearance around its colonies after a few days. *E. coli* JM83 did not form significant haloes on tributyrin plates within two weeks. This lack of hydrolytic activity in *E. coli* was exploited to isolate a complementing gene from *Acinetobacter* (further analysis of this gene led to us classifying this gene as an esterase gene; see below).

A partial *Sau3A* gene library of *A. calcoaceticus* BD413 chromosomal DNA was constructed in *E. coli* strain JM83. Chromosomal fragments, ranging in size from 5 to 10 kbp, were ligated into *Bcl*I-linearized pUN121 (Nilsson *et al.*, 1983). The ligation mixture was introduced into *E. coli* JM83 and transformants were selected on LB plates containing tetracycline and ampicillin. A total of 12000 colonies were replica-plated onto N-broth plates containing 1.5% tributyrin as an emulsion. After 4 d incubation, 12 colonies had formed a zone of clearance, indicating the production of lipolytic activity. One of these strains, JM83(pAKA10), formed zones that were significantly smaller than the haloes produced by most of the other strains. One additional strain, JM83(pAKA22), only showed a zone of clearance on tributyrin plates after 8 d incubation.

Restriction analysis showed that the first 12 strains all contained a similar 350 bp *Sa*I fragment, indicating that

they were complemented by the same gene from *Acinetobacter*. The insert in pAKA22 differed entirely from the other 12. All 13 strains were able to grow in minimal medium with 1% tributyrin as the sole carbon and energy source, although JM83(pAKA22) had a lower growth rate than the other 12 (not shown). JM83(pUN121) could not grow with tributyrin as a carbon and energy source. A similar procedure (with triacetin instead of tributyrin) was used by Reddy *et al.* (1989) to identify clones of *E. coli* that carried the esterase gene from *A. calcoaceticus* RAG-1.

Mourey & Kilbertus (1976) described tributyrin to be a lipase-specific substrate. However, McKay *et al.* (1992) have cloned an esterase gene from a psychrotrophic pseudomonad, which hydrolyses tributyrin. Since *A. calcoaceticus* BD413 produces both extracellular lipase activity and cell-bound esterase activity, the first 12 positive *E. coli* strains were replica-plated onto lipase indicator plates, to further characterize the activity cloned. No turbid haloes were formed on plates containing egg-yolk, nor did fluorescent zones appear on plates supplied with olive oil plus rhodamine B. However, as in *A. calcoaceticus* BD413, esterase activity could be detected in sonicated cell suspensions of cultures of the complemented strains, using p-NPA as a substrate. JM83(pUN121) did not show significant esterase activity (not shown). No activity was detected using the lipase substrate p-NPP (not shown). From these observations we concluded that the fragment cloned in the 12 *E. coli* strains encoded an esterase.

Mapping the esterase gene: deletion plasmids in *E. coli*

To obtain information about the molecular structure of the DNA fragment encoding the esterase activity in *E. coli*, a detailed restriction map was constructed of the plasmid inserts in pAKA10, pAKA20 and pAKA24 (Fig. 2). A 2.0 kbp fragment was present in all three plasmids, suggesting that the information necessary for esterase production was contained within this region. The restriction map of this region differed entirely from the region of the esterase gene of *A. calcoaceticus* RAG-1, cloned by Reddy *et al.* (1989). JM83 carrying pAKA10 showed a reduction in esterase production as compared to the other two strains (Fig. 2). This could indicate that either (a part of) the regulatory region in front of the esterase gene, or sequences at the end of the gene, were not present on pAKA10. The 2.8 kbp *Pvu*II/*Eco*RI fragment of pAKA24 (carrying a part of the vector), was subcloned in pUN121, digested with *Sma*I and *Eco*RI. This yielded plasmid pAKA24-5, which provided *E. coli* JM83 with the ability to degrade tributyrin, similar to pAKA20 and pAKA24 (Fig. 2), indicating that the additional 414 bp *Pvu*II/*Sau*3A fragment present in this

plasmid (in comparison with pAKA10) is important for maximal production of esterase activity.

A more detailed positioning of the esterase gene was obtained by constructing two deletion plasmids, which were subsequently used to generate the corresponding deletions in the chromosome of *A. calcoaceticus* BD413 (see below; for a more detailed description of the construction of mutants, see Methods). In the first deletion plasmid (pAKA10-1), a 2.4 kbp *HpaI*/*SmaI* fragment of pAKA10 was replaced by the *Cm^r* fragment of pMTL24p-*Cm* (Fig. 2). pAKA10-1 only contained a 1.4 kbp fragment of the esterase-encoding region. JM83(pAKA10-1) did not show a reduction in esterase activity as compared to JM83(pAKA10) (Fig. 2). Since the esterase activity in JM83 carrying either pAKA10 or pAKA10-1 was somewhat reduced as compared to JM83 carrying pAKA20, pAKA24 or pAKA24-5, the information necessary for maximal esterase expression must be located on the 1.8 kbp fragment between the *PvuII* site bordering the insert in pAKA24-5 and the *HpaI* site (Fig. 2, indicated by dotted lines).

This was confirmed by constructing a second type of deletion plasmid (pAKA20A and pAKA20B) in which the 0.34 kbp fragment between the two *SalI* sites in the 1.8 kbp region was replaced by the *lacZ*-*Km* cassette from pKOK6 in two orientations. *E. coli* JM83(pAKA20A) (Fig. 2) and JM83(pAKA20B) (not shown) did not produce esterase activity.

Esterase mutants of *A. calcoaceticus* BD413

To test whether the esterase activity in *Acinetobacter* could be abolished by deleting the wild-type gene, the deletions of pAKA20A and pAKA20B, described above, were transferred to the chromosome of *A. calcoaceticus* BD413. In addition, a second esterase deletion mutant was constructed, that lacks the entire 1.8 kbp esterase-encoding region. This mutant was constructed via a plasmid (pAKA1024-32), in which the 4.3 kbp *EcoRI* fragment present in pAKA20 was replaced by the chloramphenicol-resistance gene of pMTL24p-*Cm* (see Methods and Fig. 2).

Table 2. Maximum cell-bound esterase activities as measured in cultures of *A. calcoaceticus* strains in N-broth

Wild-type and mutant strains of *A. calcoaceticus* BD413 (see Table 1 and Methods for the construction of the mutant strains) were grown in batch culture in N-broth. Esterase activity was determined at different times during growth, in sonicated cell suspensions, using pNPA as the substrate. Maximum esterase activities were used to calculate the mean maximum esterase activity, $\pm$ SEM. *n*, Number of independent determinations.

Strain of <i>A. calcoaceticus</i>	Maximum esterase activity [U (mg protein) ⁻¹]
BD413 (wild-type)	2.3 $\pm$ 0.3 (<i>n</i> = 12)
AAC311-2	0.8 $\pm$ 0.2 (<i>n</i> = 2)
AAC312	2.6 $\pm$ 0.4 (<i>n</i> = 24)
AAC313-1/2	1.0 $\pm$ 0.1 (<i>n</i> = 5)

A. calcoaceticus AAC311-2, a deletion mutant carrying the insertion of pAKA1024-32, showed reduced esterase activity on tributyrin plates (not shown) and approximately half of the wild-type activity in *in vitro* measurements in sonicated cell suspensions (Fig. 3a; Table 2). Strains AAC313-1 and AAC313-2, deletion mutants of BD413 carrying the *lacZ*-*Km* insertions of pAKA20A and pAKA20B, respectively, showed similar reduced esterase activity in sonicated cell suspensions (Fig. 3a) and on plates (not shown). However, cell-bound esterase could still be detected in these three strains, which implies that at least one additional cell-bound esterase is produced by *A. calcoaceticus* BD413. The remaining esterase activity, like the total esterase production in BD413, was produced at the onset of the stationary phase in these mutant strains (Fig. 3a). Although the increase in production of the remaining esterase activity showed a more gradual pattern than observed for the total esterase production of BD413, this implies that both esterases in BD413 are coordinately regulated and expressed in N-broth.

At present, it is not clear whether *A. calcoaceticus* BD413 carries two (or several) copies of the same esterase gene, or entirely different esterase genes. With

Fig. 4. Detailed restriction map of the esterase-encoding region in pAKA24-5 and the strategy for sequencing fragments of this region, cloned into M13mp18 and M13mp19. The nucleotide sequence was determined using either the M13 universal primers or synthesized primers as described in Methods. The esterase ORF (*estA*) is indicated with a bold arrow. Restriction sites: *Bc*, *BclI*; *C*, *ClaI*; *EV*, *EcoRV*; *H*, *HindIII*; *Hp*, *HpaI*; *S*, *SalI*; *Sp*, *SphI*. (*Pv*), *PvuII* site lost with construction of pAKA24-5; *X*, ligation *HpaI*/*SmaI*.

<i>PvuII</i>	
CAGCTGAAGCAGGTGCTTCAACTCAAACCTGTACAGGCAGCTCCTGAAGCACACAGACAAGCCCTGATA	75
CTGCAACTGTTCAATAAGCAATAGTGACGCCTGCATCTTGAATAGTGCAGCCAAGGTAAAGTATCAAGGATGCCA	150
CCTTCACTTTACGGATTGACTCGAGCTGCTACTCAAGCAATCGCCTATTCAAGATGACATGAGCCTAAGAAATCT	225
<i>BclI</i>	
CTTACAGTCTGTGTCGAGAATGACCCTAGCTCTAGCTTAAATGATCAGAGTATGATGATTGCATACAGAACAGGA	300
TGTACTGGAATGGTTTGATTGACTTTTAAACGACATGATGTTGTTTTGCAAACCAGAGGATAAACCCCTAAATCA	375
<i>pAKA10</i>	
CCTTATGATTTAGGGGTTTATTTTATTGGAAACACTTGGATCATCCAATGTCACAAATTATTCGCAAATAATAT	450
GATAATGGTATATGGCAAAATGCGACTTCCAATCCATGTTTTTTAGTCAATGACTTTTAGGGATGATCCGATTAG	525
CGTAAATAAACACAATAATGTGACATCTTGATTGCGTACTTTAGACAACAATAGATTCAAGAACAGATAAATTGT	600

- - - - <i>estA</i> - - -	
ATCGAGAAATCAAATGACAACGACGATGAAAGGAGAGAAGAATTGACTGCATTTGTTCAAACCATTCAGGAAGTT	675
METThrAlaPheValGlnThrIleGlnGluVal	11
<i>HindIII</i>	
TTAGAAAAGGTCATGGGCCTGCTGCACGTGCTTTGGATAAGCTTCCTTCCTTCGTACAAGAGTCTATTGCGAAA	750
LeuGluLysGlyHisGlyProAlaAlaArgAlaLeuAspLysLeuProSerPheValGlnGluSerIleAlaLys	36
GTTTTAGGTTATCCCTATCAGTACCCGAGTTAGATAGCTTTATCAAATGCTTGATGGCAGTTCAAATCAAGCAA	825
ValLeuGlyTyrProTyrGlnTyrProGlnLeuAspSerPheIleLysCysLeuMetAlaValGlnIleLysGln	61
GGTCAAACCTGGGTTTATCGGCTCAGATGTGCAAAAATCAGCTTTAGCCTTTGAACTCAGATGGAGTCCATTCTT	900
GlyGlnThrGlyPheIleGlySerAspValGluLysSerArgLeuAlaPheGluThrGlnMetGluSerIleLeu	86
<i>EcoRV</i>	
CGCAAGCCCACAGCCATCACGTTTGTGCAAGATATCCGCTTACCATTACAAAGCGGAAGTATCTTTGCACGTCAT	975
ArgLysProThrAlaIleThrPheValGluAspIleArgLeuProLeuGlnSerGlyThrIlePheAlaArgHis	111
TATCATCCTGCTCCAAACAAAAAATTACCTATGATTGTGTTCTATCATGGTGGAGGATTTGTGGTGGGCAATGTT	1050
TyrHisProAlaProAsnLysLysLeuProMetIleValPheTyrHisGlyGlyGlyPheValValGlyAsnVal	136
<i>HindIII Sall</i>	
GATACACATGATGAAGCTTGTGCGACTCATTGCAAAATACGCCAATGCTCAAGTATTAAGTATCGATTATCCATTG	1125
AspThrHisAspGluAlaCysArgLeuIleAlaLysTyrAlaAsnAlaGlnValLeuSerIleAspTyrProLeu	161
GCACCAGAAGTTTCACCGCAACGACTAATTCAGTCTTGTGAAGATGCCTTAGCTTGGGTTTATCAAACAAACGT	1200
AlaProGluValSerProGlnArgLeuIleGlnSerCysGluAspAlaLeuAlaTrpValTyrGlnAsnLysArg	186
CATTTTAAATACTAAAAATCAGATTGCCGTGGCAGGTGATAGTGCGGGTGGAATATCAGCACCGTTGTGGCT	1275
HisPheLysIleLeuLysAsnGlnIleAlaValAlaGlyAspSerAlaGlyGlyAsnIleSerThrValValAla	211
CAGCGTGCATTTGGAAGTTTACGCACAAGATGCACAATTCCTGATTTATCCAGTAGTTGATTTTAAAGCCGT	1350
GlnArgAlaIleGlyLysValTyrAlaGlnAspAlaGlnPheLeuIleTyrProValValAspPheLysSerArg	236
<i>Sall</i>	
CATCCCTCATTTTATGCATATAAAGATGGATTGGTGCTGACTGGAAATGACGTCGACTATGTGACTGATTACTAT	1425
HisProSerPheTyrAlaTyrLysAspGlyLeuValLeuThrGlyAsnAspValAspTyrValThrAspTyrTyr	261
<i>SphI</i>	
GCAACAAAGCATGCTGTACATTTAGATGATCCAATTATTTGCGCTACCTATGGTAATTTCAAAAACTGGCGCCT	1500
AlaThrLysHisAlaValHisLeuAspAspProIleIleSerProThrTyrGlyAsnPheLysLysLeuAlaPro	286
GCATATATTGTGACGGCTGGACATGATGTATTACATGATGAAGGCGAAATCTATAGCCATAAGCTACGTCAAGCA	1575
AlaTyrIleValThrAlaGlyHisAspValLeuHisAspGluGlyGluIleTyrSerHisLysLeuArgGlnAla	311
<i>BclI</i>	
GGGGTTAAATTCATTTTGAAGAGTACCTTGATCAAACCCATGGATTATCAATTTGACGCCAGTTTCACATAAG	1650
GlyValLysIleHisPheGluGluTyrLeuAspGlnThrHisGlyPheIleAsnLeuThrProValSerHisLys	336
GCGAGAGCAAATCTGATTTCAGATGAGTAAATCATTCGGTAAATTTTGAATAAATACGCCCTGATATTAAGATCAG	1725
AlaArgAlaAsnLeuIleGlnMetSerLysSerPheArgLysPheTrpAsnLysTyrAla***	356
TTGAAATTTGATACTATTTGAAGAAATAGTATCAAATACACAATTAACAAGAGAGTAGACAGTTTATGCTATG	1800
<i>HpaI</i>	
TTAAGTCACTTATGATAAAAGAAGTTAAC	1829

respect to this, it is relevant to note the identification of *E. coli* JM83 strain carrying pAKA22 (see above). The suggestion that the insert in pAKA22 encodes a second esterase from *A. calcoaceticus* is currently under investigation.

To our knowledge, the presence of two esterases has not been reported for other strains of *Acinetobacter*. It seems different from the situation described for *A. calcoaceticus* RAG-1 by Shabtai & Gutnick (1985): Mutants of RAG-1, defective in an esterase, were unable to grow on triacetin, whereas they could still grow on acetate. Transfer of the deletion of pAKA10-1 (which had no influence on the esterase production in JM83) to *Acinetobacter* yielded strain AAC312. As expected, this deletion mutant exhibited the full cell-bound esterase activity as found in BD413 (Table 2), showing that also in *Acinetobacter* the 2.4 kbp *HpaI*/*SmaI* region is not essential for the production of the esterase.

The production of extracellular lipase activity in the *A. calcoaceticus* strains mentioned above was not affected by the mutations in the esterase region (Fig. 3b). This shows that the two activities are not genetically related.

Sequence analysis of the esterase encoding region

The nucleotide sequence of the 1.8 kbp *PvuII*/*HpaI* fragment of pAKA24-5 was determined. The strategy for sequencing of this fragment is shown in Fig. 4, the nucleotide sequence and derived amino acid sequence in Fig. 5. The 1829 bp fragment contains one open reading frame (ORF), from nucleotide 580 to 1710. The ORF is followed by a UGA and a UAA stopcodon, separated by only three bases. The ORF was termed *estA*, because of the esterase activity it encodes (see above).

The first ATG codon in the ORF is located at nucleotide position 805. If translation is initiated from this methionine codon, a stretch of 225 nucleotides of potentially coding DNA remains untranslated. The ORF would then encode a protein of 302 amino acids, with a calculated molecular mass of 33900 Da. However, the ATG codon is not preceded by a ribosome binding site (RBS), i.e. a sequence complementary to the 3' end of the 16S rDNA of *A. calcoaceticus* (5'-GATCACCTCCTT-3'; C. R. Woese, EMBL data library, acc. no. M34139; Fig. 5). An alternative translation start site in the same reading frame is the TTG codon at position 643.

Although a UUG codon is thought to be less efficient in initiation of protein synthesis, the TTG codon is preceded by a potential RBS (⁶³²GGAG), with a calculated free energy for binding to the 3' end of *A. calcoaceticus* 16S rRNA of $-39.3 \text{ kJ mol}^{-1}$ (Tinoco *et al.*, 1973), and a spacing of 7 nucleotides. This TTG codon may therefore be a good alternative to start translation of the esterase gene (Thach *et al.*, 1966; Kozak, 1983; Hershey, 1987). If translation is initiated from this position, the protein encoded contains 356 amino acids, and has a predicted mass of 40.0 kDa.

The actual mass of the esterase has not yet been determined. However, the translation start site has been determined via *lacZ* fusion strain AAC313-1, in which the *estA* ORF has been fused in-frame to a promoterless *lacZ* gene (see Methods and below, *Measurements of β -galactosidase in Acinetobacter lacZ fusion strains*). The mass of the fusion product has been measured on Western blots, incubated with polyclonal rabbit antibodies raised against β -galactosidase. When the ⁸⁰⁵ATG is used as the start codon, the mass of the EstA- β -galactosidase fusion product should be increased by roughly 10 kDa, relative to the native β -galactosidase, since the fusion was made at the ¹⁰⁷¹*SalI* site. Translation initiated from the ⁶⁴³TTG should result in an increase in mass of about 16 kDa. A fusion product was found with a mass increase of about 17 kDa relative to the native β -galactosidase, identifying the ⁶⁴³TTG as the true translation start codon. The *lacZ* gene used carries its own RBS. Therefore, apart from the fusion product, native β -galactosidase can also be formed in AAC313-1. In fact, relatively small amounts of the EstA- β -galactosidase product were found; the main product formed was the native enzyme, as judged by the relative staining intensity of bands on Western blots. This may be due to the relative inefficiency of TTG as a translation start codon.

Codon usage and codon base composition of the esterase gene are similar to what has been calculated as the average for 20 *Acinetobacter* genes by White *et al.* (1991). The esterase gene has a combined A+T composition at codon positions one, two and three of 44.9, 61.6 and 66.3 mol %, respectively, exactly matching calculations by White *et al.* (1991). The overall A+T content of the esterase gene (60 mol %), is also in agreement with the A+T content of the *Acinetobacter* genome (55–62 mol %; Henriksen, 1976).

Fig. 5. DNA sequence of the 1.83 kbp *PvuII*/*HpaI* chromosomal insert in pAKA24-5 encoding an esterase, and predicted amino acid sequence of EstA. The nucleotide sequence was determined via the strategy outlined in Fig. 4. The single large ORF on the nucleotide sequence is underlined. The amino acid sequence derived from this ORF is given, starting from the TTG codon at nucleotide position 643. The potential ribosome-binding site is doubly underlined. Relevant stop codons are marked ***. The two large inverted repeats are overlined. Nucleotides and derived amino acids are numbered to the right. Relevant restriction sites are given above the sequence; 'pAKA10' indicates the *Sau3A* site at nucleotide position 414, bordering the insert in pAKA10 (see text).

Table 3. Homology of protein sequences, extracted from data libraries, to *A. calcoaceticus* BD413 EstA

Data were obtained from the Swiss-Prot and PIR (only human carboxylesterase) data libraries with the sequence comparison program FastA (Pearson and Lipman, 1988). Proteins are only included if they show homology to EstA in an overlap of more than 100 amino acids. When no reference was cited in the data library, only the first author is given, together with the accession number from the data library.

Protein homologous to BD413 EstA (organism)	Percentage of amino acids identical to EstA (no. of overlapping amino acids)	Reference and/or accession no.
Lipase (<i>Moraxella</i> TA144)	37.2% (113)	Feller <i>et al.</i> (1991)
Hypothetical protein (<i>E. coli</i>)	38.5% (104)	Miyamoto <i>et al.</i> (1991)
Esterase (<i>A. calcoaceticus</i> RAG-1)	19.1% (262)	Reddy <i>et al.</i> (1989)
Lipase (rat)	28.7% (164)	Holm <i>et al.</i> (1988)
Vegetative specific protein H5 (<i>Dictyostelium</i>)	21.4% (252)	Singleton <i>et al.</i> (P14326)
N-Acetylphosphinothricin-tripetide-deacetylase (<i>Streptomyces viridochromogenes</i>)	25.1% (231)	Alijah <i>et al.</i> (S20686)
Carboxylesterase precursor (mouse)	26.1% (134)	Ovnik <i>et al.</i> (1991)
Liver carboxylesterase precursor (man)	27.6% (134)	Long <i>et al.</i> (P23141)
cDNA encoding a liver carboxylesterase (man)	26.8% (123)	Riddles <i>et al.</i> (1991)
Proline- β -naphthylamidase (domestic pig)	26.1% (134)	Matsushima <i>et al.</i> (1991)
Liver carboxylesterase 1 precursor (rat)	21.2% (165)	Takagi <i>et al.</i> (1988)
Juvenile hormone esterase precursor (<i>Heliothis virescens</i>)	22.3% (139)	Hanzlik <i>et al.</i> (1989)
Liver carboxylesterase 1 precursor (rabbit)	27.0% (126)	Korza & Ozols (1988)
Liver carboxylesterase 2 precursor (rabbit)	24.1% (133)	Ozols (1989)

The amino acid sequence deduced from the ORF shows homology to several proteins from the PIR and Swiss-Prot data libraries, as determined with the sequence comparison program FASTA (Pearson & Lipman, 1988). The homologous proteins are mainly esterases and some lipases (Table 3). In Fig. 6, the amino acid sequence of the BD413 protein (BD413 EstA) is aligned to the four proteins with the highest degree of homology, using the multiple sequence analysis program PileUp (see Methods). Although homology of the BD413 EstA sequence to the *A. calcoaceticus* RAG-1 esterase (P18773; Reddy *et al.*, 1989) is found in a relatively large overlapping region (Table 3), it is clear from Fig. 6 that specific regions of EstA are more homologous to the other three proteins. For instance, the ¹²⁷HGGGF sequence found in BD413, also present in all eukaryotic esterases of Table 3 (not shown), is one of the strikingly homologous regions in the sequences of the upper four proteins of Fig. 6, and is not found in the RAG-1 esterase. Some other amino acids, identical in all but the RAG-1 esterase, are also indicated in Fig. 6.

Several amino acids are conserved in all five proteins aligned in Fig. 6. The sequence ¹⁹⁹GDSAG in the BD413 esterase is also found in the lipases of *Moraxella* and rat. The RAG-1 esterase also contains this box, but it contains a cysteine instead of an alanine. This sequence is identical to the Gly-X₁-Ser-X₂-Gly box found in lipases and esterases, as well as in serine proteinases (Brenner, 1988; Boel *et al.*, 1988). The serine in this sequence is

probably the nucleophilic residue in the catalytic triad of these enzymes, consisting of an additional histidine and aspartate (Blow, 1990; Brady *et al.*, 1990; Winkler *et al.*, 1990). Although Boel *et al.* (1988) showed X₁ to be either tyrosine or histidine in a number of prokaryotic and eukaryotic lipases, the sequences in Fig. 6 all have an aspartate at this position. Also several other residues have been described to occur at the X₁ and X₂ positions (Brenner, 1988; Dartois *et al.*, 1992). Interestingly, like the lipolytic enzymes in Fig. 6, almost all serine proteases contain an aspartate at the X₁ position. However, in these enzymes the X₂ position is almost invariably a glycine (Brenner, 1988). A histidine (position 85 in EstA) and an aspartate residue (position 104 in EstA) are found in all five proteins, but whether these are the other two residues of the catalytic triad (Blow, 1990), still has to be confirmed for EstA.

No promoter-like sequences (Hoopes & McClure, 1987) could be identified in the sequence upstream of the esterase ORF. However, two large inverted repeats were found in the sequence of the *PvuII/HpaI* fragment. The first one is located upstream of *estA*, between nucleotides 360 and 396, potentially forming a perfect stem-loop structure, with a stem of 16 nucleotides and a loop of five. This structure has a strongly negative free energy ($\Delta G = -120.4 \text{ kJ mol}^{-1}$; Tinoco *et al.*, 1973). The program 'Terminator' identified this sequence (or part of it) as a possible rho-independent terminator for transcription (Rosenberg & Court, 1979), starting from the

BD413 EstA	-----IVFY	HGGG	FVVGNV DTHDEACRLIAKYANAQVLS	156		
P24484	-----ERKSTDEAAMLFF	HGGG	FCIGDIDTHHEFCHTVCAQTGWAVVS	194		
P23872	-----CPQPDSPATLFYL	HGGG	FILGNLDTHDRIMRLLASYSQCTVIG	120		
P15304	LAKSEGPRLELRPRPQQAPRSRALVVHI	HGGG	FVAQTSKSHEPYLKNWAQELGVPIIS	378		
P18773	STNSVWAGGTVGEELSAQSATELILRLF	MAVD	F-LGRLNTHRALMTDPLARASDEVIIH	90		
***.....*						
BD413 EstA	IDYPLAPEVSPQRLIQSCEDALAWVYQNKRFKILKNQIAVAGDS	A	GGNISTV	V	AQ	212
P24484	VDYRMAPEYPAPTALKDCLAAYAWLAHESQSLGASPSRIVLSGDS	A	GGCLAAL	V	AQ	250
P23872	IDYTLSPPEARFPQAIEEIVAACCFYHQQAEDYQINMSRIGFAGDS	A	VPCWRSP	V	R-	175
P15304	IDYSLAPEAFPFRALIEECFFAYCWAHVHCELLGSTGERICLAGDS	A	GGNLCIT	V	SL	434
P18773	VDYPLAPEHPYPEAIDVIFDGLSSTTRS	----	RVKPKDIIISGDS	C	GANLHLA L SL	142
.						
BD413 EstA	RAI-----GKVYA--QD	-----	AQFLIYPVVDFKSRH	237		
P24484	QVI-----KPIDALWQDNNQAPAADKKVNDTFKNSLADLPRPLAQPLYPVTDYEAAY	-----	CGCVINRSIA	185		
P23872	-----	-----	CGCVINRSIA	185		
P15304	RAAAYGVRVPDGIMAAYPVTTLOSSASPSRLLSLMDPLLPLSVLSKCVSAYSGTETEDHF	-----	LKQQPELMPEWFDIDVALFGSDTDPVNRY	494		
P18773	R-----	-----	LKQQPELMPEWFDIDVALFGSDTDPVNRY	172		
.						
BD413 EstA	PSFYAYKDGLVLTGNDVDYV-----	TDYYATKHAVHLDDPIISPTYGNFKKLAPAYI	289			
P24484	PSWELYGEGLLLDHNDAEVF-----	NSAYTQHSGLPQSHPLISVMHGDN TQLCPSYI	355			
P23872	VKLRAFCCGMGFTDYGIP-----	-----	203			
P15304	DSDQKALGVMGLVQRDTSLFLRDLRLGASSWLNSFLELSGRKPKHTPVACNRDTAPHGFW	-----	554			
P18773	VSIRSTMHCYRLKALQAGI-----	KHYLTDDIQPGDPRVSPLFDDLDGLPPTLV	221			
.						
BD413 EstA	V-----TAGHDVLHD	EG	EIYSHK	L	RQAGVKIHFEYLDQTHGFINLTPVSHKA	337
P24484	V-----VAELDILRD	EG	LAYAEL	L	QKEGVQVQTYTVLGAPHGFINLMSVHQGL	403
P23872	-----	---	---	---	-----	---
P15304	ALTESMRRSVSEAAALAP	EG	LLGTDS	L	KKLTIKDLSFKGNSEPSDSPEMSQSMETL	610
P18773	-----QVGSKEIQD	DS	KGFREK	A	EQADVKVHFKLYTGMWNNFQMF-----	262
.						
BD413 EstA	RANLIQMSKS	F	RKFWNKYA	-----	356	
P24484	GNQTTYIINE	F	ACLVQNLLTSEGDKPNLRA	-----	433	
P23872	-----	---	---	---	-----	---
P15304	GPSTPSDVNF	F	LRSGNSQEEAETRDDISPMDGIPRVRAAFPDPGFHPRRSSQGVLMHPL	668		
P18773	--NAWEFANR	Y	LDYLDQISKMHVCRDRCHS	-----	290	

Fig. 6. Alignment of the predicted amino acid sequence of EstA (BD413 EstA), with the four proteins with the highest homology to EstA. Only part of the amino acid sequences is shown. Data were obtained from the Swiss-Prot protein sequence data library. The numbers at the beginning of each line are the accession numbers of the proteins in the Swiss-Prot library: P24484 is the lipase of *Moraxella* (Feller *et al.*, 1991), P23872 is the *E. coli* hypothetical protein (Miyamoto *et al.*, 1991), P15304 is the rat lipase (Holm *et al.*, 1988) and P18773 is the esterase of *A. calcoaceticus* RAG-1 (Reddy *et al.*, 1989). Homology is indicated above the amino acid sequences; asterisks denote amino acids perfectly conserved in all five proteins and dots denote well-conserved amino acids. Amino acid residues perfectly conserved in all but the RAG-1 esterase are shown between vertical lines. Gaps in the sequences, created by the program, are dashed. Numbers to the right refer to the number of the last amino acid residue on the line, relative to the first amino acid of the protein. Carboxy-terminal amino acids are underlined.

upstream region of *estA*. However, if the inverted repeat sequence between positions 360 and 396 is a terminator for transcription, the distance between it and the end of upstream ORFs is rather long (at least 262 nucleotides).

Interestingly, restriction analysis showed that the insert in pAKA10 starts at position 414 of the sequence in Fig. 5, just downstream of the inverted repeat. Since the level of esterase activity in *E. coli* JM83(pAKA10) is reduced in comparison with strains carrying the larger insert, it is tempting to speculate about the possible role of the 360–396 inverted repeat in regulation of *estA*. This

structure, or other sequences on the 414 bp *PvuII/Sau3A* fragment, may carry promoter functions or form a binding site for a regulatory protein, involved in regulation of *estA* transcription (Hoopes & McClure, 1987).

Speculation about the possible role of the 360–396 inverted repeat in the regulation of *estA* expression is also fed by the identification of a second large inverted repeat, located 27 nucleotides downstream of the *estA* ORF (Fig. 5). This sequence, between nucleotides 1731 and 1762, may form a perfect stem-loop, with a stem of

Fig. 7. Production of β -galactosidase by *lacZ* fusion strains of *A. calcoaceticus*. Growth (OD_{540} ; ●) and β -galactosidase activity (Miller units) were measured in batch cultures of AAC313-1 (△) and AAC313-2 (▽), grown in N-broth in well-aerated fermenter vessels. Only the growth curve of AAC313-1 is shown.

14 nucleotides and a loop of four. The structure has a free energy of $-65.2 \text{ kJ mol}^{-1}$. Unlike the inverted repeat upstream of the esterase gene, this sequence has no strong similarity to transcription terminators. It lacks the poly-T stretch of nucleotides, normally following the inverted repeat, which serves an important function in rho-independent transcription termination (Rosenberg & Court, 1979). Also in this case, the structure may be involved in the regulation of gene expression, e.g. of an open reading frame downstream of *estA*.

The possible regulatory role of the two inverted repeats found in the esterase-encoding fragment, together with the function of a potential downstream ORF, are now under investigation.

Measurements of β -galactosidase in *Acinetobacter lacZ* fusion strains

The *lacZ*-Km cassette of pKOK6, inserted in two orientations between the two *SalI* sites within the esterase ORF in strains AAC313-1 and AAC313-2, carries a promoterless *lacZ* gene. β -Galactosidase activity was measured in washed cells of these strains grown in N-broth, to confirm the direction of transcription of the esterase gene. Furthermore, the *lacZ* fusion strains could aid in unravelling the regulation of the expression of *estA*.

In Fig. 7, the production of β -galactosidase is shown during growth of AAC313-1 and AAC313-2 in a batch culture in N-broth. No β -galactosidase activity could be detected in AAC313-2. However, mutant AAC313-1, carrying the *lacZ* gene in the same direction as the predicted esterase ORF, did show β -galactosidase activity. Moreover, like the esterase, the β -galactosidase was produced at increasing levels during the onset of the stationary phase. Interestingly, in this recombinant strain,

a constant level of 75 Miller units β -galactosidase was produced during exponential growth. A sharp increase in β -galactosidase level was observed within 1.5 h after the culture reached the stationary phase of growth. This was followed by a slow increase in the production of β -galactosidase, up to twice the level produced in the exponential phase.

The β -galactosidase measurements of Fig. 7, together with the detection of an *EstA*- β -galactosidase fusion protein (see above), confirm the direction of transcription predicted by the sequence of the esterase region. Furthermore, the time dependence of β -galactosidase production in AAC313-1 shows that production of the esterase is regulated at the genetic level. This may be regulation of transcription only, but the possible involvement of growth-phase-dependent mRNA (in)stability and/or regulation at the level of translation cannot be ruled out at this stage.

The molecular mechanism underlying the regulation of *estA* expression is currently being investigated in *lacZ* fusion strains, carrying partial deletions of the upstream region of *estA*. The physiological factors involved in transcriptional regulation of the esterase gene will be analysed, as well as the regulation of the production of the extracellular lipase and the second cell-bound esterase.

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