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Translocation of *Vibrio harveyi N,N'*-Diacetylchitobiase to the Outer Membrane of *Escherichia coli*

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The gene encoding *N,N'*-diacetylchitobiase (chitobiase) of the chitinolytic marine bacterium *Vibrio harveyi* has been isolated. While expression of the chitobiase gene (*chb*) was inducible by *N,N'*-diacetylchitobiose in *V. harveyi*, it was expressed constitutively when cloned in *Escherichia coli*, suggesting that controlling elements are not closely linked to *chb*. Chitobiase was found in the membrane fraction of *E. coli* cells containing plasmids with the cloned *V. harveyi chb* gene. When membranes of such cells were separated on Osborn gradients, chitobiase activity was found mainly in the outer membrane band. Translocation of the enzyme to the outer membrane was accompanied by cleavage of a signal peptide. A fusion protein, in which 22 amino acids from the amino terminus of prechitobiase were replaced with 21 amino acids from the pUC19 *lacZ* amino terminus, was not processed, and 99% of the activity was located in the cytoplasmic fraction. A homology to six amino acids surrounding the lipoprotein processing and modification site was found near the amino terminus of prechitobiase.

Chitin is an insoluble polysaccharide consisting of β-(1,4)-linked *N*-acetylglucosamine (GlcNac) units. Large reservoirs of this polysaccharide in the marine and terrestrial environments are derived from the cell walls of fungi and the cuticles of crustaceans and insects (9, 23). Many bacteria in these environments are able to hydrolyze chitin to GlcNac (16, 18, 19) by using two enzymes, chitinase and *N,N'*-diacetylchitobiase (chitobiase). Chitin is first hydrolyzed by chitinase to low-molecular-weight multimers of GlcNac, the dimer *N,N'*-diacetylchitobiose (chitobiose) being predominant. Chitobiase then hydrolyzes chitobiose to GlcNac.

To begin an analysis of these enzymes, we have cloned the genes encoding chitinase and chitobiase from the gram-negative marine bacterium *Vibrio harveyi*. Chitinase genes have also been cloned from *Serratia marcescens* (5, 10), in which two chitinase genes encoding two different chitinases have been identified (10). The chitobiase gene has been isolated from *S. marcescens* (8) and *Vibrio vulnificus* (25). Here, we show that chitobiase is located in the outer membrane of *Escherichia coli* cells containing the cloned chitobiase gene (*chb*). Based on minicell analysis of plasmid-encoded proteins, the approximate sizes of the unprocessed and processed proteins are 95.3 kilodaltons (kDa) and 92.1 kDa, respectively. When the first 21 amino acids of *lacZ* from pUC19 were substituted for the first 22 amino acids of prechitobiase, a hybrid protein resulted that had chitobiase activity, remained unprocessed, and was found only in the cytoplasm, as would be expected if the amino terminus of prechitobiase were involved in processing and translocation to the outer membrane.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* K-12 strain LE392 (F^-) *hsdR lacY galK2 galT22 metB1 trpR55 supE44 supF58*, *E. coli* K-12 strain JM109 (F' *traD36 proAB*$^+$ *lacI*^{q}*ZΔM15*) *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*), the *E. coli* K-12 minicell strain p678-54 *thr ara leu azi fhuA lacY tsx minA minE gal malA thi xyl rpsL*, and *V. harveyi* B392 (21) were used in these studies. Plasmids pMK2004 (11) and pUC19 (26) were used as vectors. Cells were grown in minimal alkaline phosphatase medium [0.1% $(NH_4)_2SO_4$, 0.05% sodium citrate, 100 mM Tris chloride (Sigma Chemical Co.), pH 7.4, 0.02% $MgSO_4$, 0.5% Casamino Acids (Difco Laboratories), 0.3% glycerol, 50 μM K_2HPO_4, 0.005% thiamine, and 40 μg of amino acids per ml as required], L broth (16), or LM broth (L broth with 2.0% NaCl). For plasmid selection, 50 μg of kanamycin (Sigma) per ml or 100 μg of ampicillin (Sigma) per ml was added to the medium. Transformation was performed as described previously (4).

Isolation of plasmid DNA. The alkaline lysis method (1) was used for routine examination of plasmid DNA, and the cleared lysate method (3) was used when larger amounts of more highly purified plasmid DNA were needed and included two successive isopycnic ethidium bromide-cesium chloride gradient centrifugations.

Enzymes, assays, and gel electrophoresis. Restriction endonucleases were obtained from Bethesda Research Laboratories (BRL), New England Biolabs, or Boehringer Mannheim, and T4 DNA ligase was from BRL. In all cases, the reaction conditions recommended by the manufacturers were used. The molecular weights of plasmids and restriction fragments were determined by electrophoresis on 0.7% to 2.0% horizontal agarose (BRL) slab gels in Tris acetate-EDTA buffer (6).

Chitobiase activity was determined with either *p*-nitrophenyl-*N*-acetyl-β-D-glucopyranoside (PNAG) or chitobiose as the substrate. With PNAG as the substrate (method 1), the assays were conducted as described previously (R. W. Soto-Gil, L. C. Childers, W. H. Huisman, A. S. Dahms, M. Jannatipour, F. Hedjran, and J. W. Zyskind, Methods Enzymol., in press) in 10 mM Tris chloride (pH 7.3)–666 μM PNAG in a volume of 1 ml at 37°C. Enzyme samples were added at time zero to each of the reaction mixtures, which were subsequently incubated for 3 to 15 min before termination of the reaction by the addition of 3 ml of 1 M Tris

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TABLE 1. Effect of chitobiose on expression of chitobiase activity[a]

Bacterial strain	Plasmid	Chitobiose	Chitobiase activity[b] (U)	
			0 min	60 min
V. harveyi B392	None	−	<0.07	<0.07
	None	+		0.37
E. coli LE392	pRSG16	−	0.86	0.58
	pRSG16	+		0.66
	pRSG14	−	2.93	2.89
	pRSG14	+		2.39

[a] *V. harveyi* cells were grown in LM broth at 30°C and *E. coli* cells in L broth containing kanamycin (50 μg/ml) at 37°C. At an OD_{450} of 0.2 (time zero) chitobiose (0.2 mM) was added to half of the culture (+) and incubated together with the other half (−) for an additional 60 min.

[b] Units per milliliter of culture per OD_{450} unit. The activity of chitobiase was determined by method 1 with PNAG as the substrate.

base, resulting in a pH of >10.5. *p*-Nitrophenol release was measured immediately at 400 nm with a molar absorptivity of 1.8×10^3 liters mol^{-1} cm^{-}, and control values for mixtures lacking enzyme were subtracted from assay values. One unit of chitobiase activity in this assay is defined as the amount of enzyme that catalyzed the formation of 1 μmol of *p*-nitrophenol in 1 min at 37°C.

In the other procedure (22; Soto-Gil et al., in press) used to assay chitobiase, *N,N'*-diacetylchitobiose (chitobiose) was the substrate (method 2). Samples to be assayed were added to 10 mM Tris chloride, pH 7.3, in a total volume of 1 ml. After preincubation at 37°C for 5 min, 0.5 ml of 2 mM chitobiose in the same buffer was added to initiate the reaction, and incubation at 37°C was continued for 3 to 15 min. The sample was then boiled for 10 min to stop the reaction, and the amount of GlcNac released was measured by using the *p*-dimethylaminobenzaldehyde reagent (Aldrich) as described previously (Soto-Gil et al., in press). One unit of activity in this assay is defined as the amount of enzyme that catalyzed the formation of 1 μmol of GlcNac in 1 min at 37°C.

Alkaline phosphatase was assayed as described (7). One unit of alkaline phosphatase is equivalent to the amount of enzyme that released 1 μmol of *p*-nitrophenol per min at 37°C with the artificial substrate *p*-nitrophenyl phosphate (Sigma). The assay for β-galactosidase was performed as described by Miller (15). One unit of β-galactosidase activity is defined as the amount of enzyme that released 1 μmol of *o*-nitrophenol per min at 28°C from *o*-nitrophenyl-β-D-galactopyranoside (Sigma).

Induction studies. Overnight cultures of *V. harveyi* B392 grown in LM broth and *E. coli* LE392 containing pRSG14 or pRSG16 grown in L broth were diluted 1:100 in the same medium and incubated to an OD_{450} of 0.2. Chitobiose was added at a final concentration of 0.2 mM to half of each culture, and all cultures (with and without chitobiose) were incubated at their optimal growth temperatures (30°C for *V. harveyi*, 37°C for *E. coli*). At 0 and 60 min (Table 1), the OD_{450} of the culture was measured, and duplicate 1-ml samples were pipetted into test tubes in ice containing 10 μl of chloramphenicol (20 mg/ml in ethanol). The cells were washed twice with M9 salts (15) (containing 1.5% NaCl for *V. harveyi*), and chitobiase activity was assayed after removal from the outer membrane with 1/10 volume of 1% *N*-lauroylsarcosine (Sarkosyl; Sigma) followed by incubation at 37°C for 1 h as described previously (Soto-Gil et al., in press). The cells were removed by centrifugation, and the remaining supernatant was assayed for chitobiase activity by method 1 with PNAG as a substrate.

Minicell analysis of plasmid-encoded proteins. Plasmids were introduced into strain P678-54 by transformation, and minicells were isolated following the previously described procedure (14) with the following modifications. The sucrose gradients were formed by freeze-thawing a 20% sucrose solution in buffered saline gelatin (85% NaCl, 0.03% KH_2PO_4, 0.06% $NaHPO_4$, and 100 μg of gelatin per ml), and the minicells were purified through three successive sucrose gradients. The concentration of minicells was adjusted to an OD_{450} of 0.5 to 0.7 with a solution containing minimal M9 salts, 0.5% glycerol, and 2 μg of amino acids (minus methionine) per ml. The minicells were incubated at 37°C with shaking for 1 h in the presence of D-cycloserine (50 μg/ml) to inhibit growth of whole cells, and then 15 μCi (1,000 Ci/mmol) of [^{35}S]methionine (Amersham) was added to 1 ml of minicells, and incubation was continued at 37°C for another 1 h. After the addition of 500 μl of L broth, the minicells were pelleted, suspended in 20 μl of SDS-lysing buffer (10 mM Tris chloride, pH 6.8, 2% sodium dodecyl sulfate [SDS], 10 mM $MgCl_2$), and heated for 2 min at 95 to 100°C. The preparations were stored at −70°C or used directly. Proteins were separated on 9% polyacrylamide gels as described (13). After electrophoresis, the gels were soaked in a solution of Amplify (Amersham) for 30 min, dried, and exposed to preflashed Kodak XAR-5 film for 40 h.

Separation of *E. coli* cells into extracellular, periplasmic, cytoplasmic, and membrane fractions. The method of cell fractionation was based on the procedure of Koshland and Botstein (12). Cells containing either pRSG192 or pRSG196 were grown to an OD_{450} of 0.5 in 15 ml of minimal alkaline phosphatase medium containing ampicillin (200 μg/ml) and 1 mM isopropylthiogalactoside (IPTG). Cells were concentrated by centrifugation, and all further manipulations were performed at 4°C. The culture supernatant (extracellular fraction; Table 2) was saved, and the pellet was suspended in 1.13 ml of 0.1 M Tris chloride (pH 8.0)–0.5 mM EDTA–0.5 M sucrose. Lysozyme was added (120 μl of a 2-mg/ml solution), followed by the addition of 1.13 ml of cold distilled H_2O. The sample was incubated for 25 min on ice, and the process of removing the cell wall was monitored by phase-contrast microscopy. As soon as the majority of cells had become spheroplasts, 45 μl of 1 M $MgCl_2$ was added to stabilize the spheroplasts. After centrifugation, the supernatant was saved as the periplasmic fraction (Table 2). The residual fluid was removed, and the remaining pellet was rapidly suspended in 1.5 ml of cold H_2O. Following lysis of the spheroplasts in the hypotonic solution, the mixture was centrifuged for 10 min at 1,000 × *g* to remove intact cells. An 0.5-ml portion of the supernatant was saved as the cytoplasmic plus membrane fraction (Table 2), and 1.0 ml was diluted with 4.0 ml of H_2O and centrifuged at 100,000 × *g* for 2 h. The pellet was saved as the membrane fraction, and the supernatant was saved as the cytoplasmic fraction (Table 2).

Separation of the membrane into inner and outer fractions. Cytoplasmic and outer membrane fractions were separated by isopycnic sucrose gradient centrifugation (20) after labeling phospholipids with [^{3}H]glycerol. Cells were grown in L broth (200 ml) at 37°C to an OD_{600} of 0.6 to 0.8 in the presence of ampicillin (200 μg/ml) and 2 μM [2-^{3}H]glycerol (1 mCi/μmol), followed by centrifugation at 13,000 × *g* for 5 min at 4°C. The cell pellet was suspended in 12 ml of cold 0.75 M sucrose–10 mM Tris chloride buffer, pH 7.8. Lysozyme was added to a final concentration of 100 μg/ml, and after incubation on ice for 2 min, the mixture was slowly

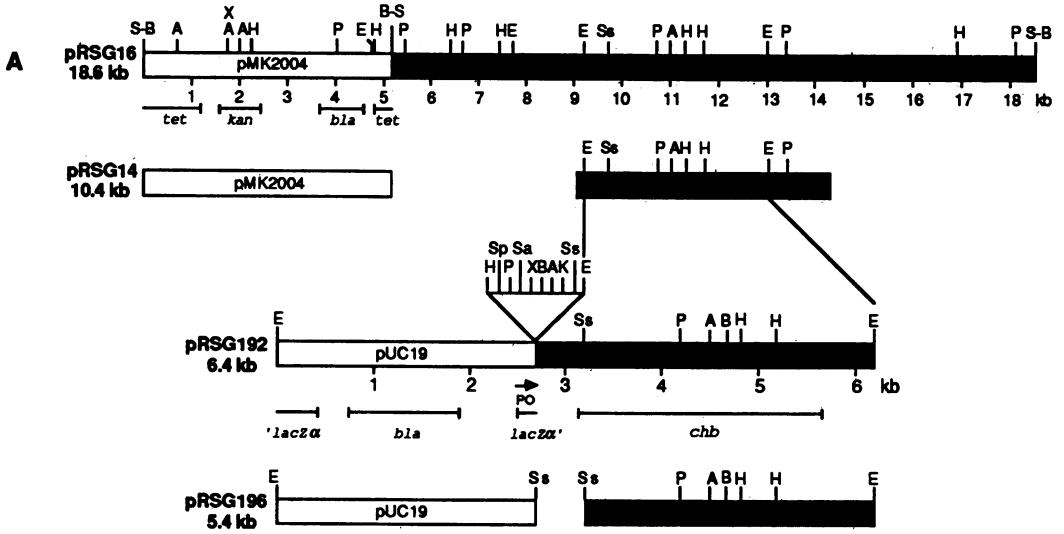
TABLE 2. Enzymatic activities of cellular fractions of *E. coli* LE392 containing pRSG192 or pRSG196

Cell fraction	Chitobiase[a]		β-Galactosidase		Alkaline phosphatase	
	Activity[b] (U)	% of total activity	Activity (U)	% of total activity	Activity (U)	% of total activity
pRSG192						
Extracellular	<0.1	<1	<0.1	<1	37.0	47
Periplasmic	1.5	3	0.1	2	37.9	48
Cytoplasmic plus membrane	61.3	97	5.7	98	4.2	5
pRSG196						
Extracellular	<0.1	<1	0.2	3	37.0	34
Periplasmic	1.5	2	0.4	5	50.5	48
Cytoplasmic plus membrane	74.5	98	6.5	92	18.5	18
pRSG192						
Cytoplasmic	14.5	33	5.7	89	ND[c]	ND
Membrane	29.3	67	0.7	11	ND	ND
pRSG196						
Cytoplasmic	66.5	99	12.0	98	ND	ND
Membrane	0.9	1	0.3	2	ND	ND

[a] Activity of chitobiase was determined by method 1 with PNAG as the substrate.
[b] Activity represents total units of activity per fraction except for cytoplasmic-plus-membrane, cytoplasmic, and membrane fractions, which were normalized to 1.5 ml.
[c] ND, Not determined.

diluted with 24 ml of cold 1.5 mM EDTA, pH 7.5. After conversion of spheroplasts occurred, lysis was facilitated by the addition of 144 ml of cold H_2O, followed by stirring for 10 min at 4°C. Lysates were centrifuged for 20 min at 1,200 × *g* to remove intact cells, and the supernatant was centrifuged at 360,000 × *g* for 2 h at 4°C. The membrane pellet was suspended in 60 ml of cold 0.25 M sucrose–1 mM EDTA–3.3 mM Tris chloride, pH 7.6. The membranes were again centrifuged for 2 h as above and suspended as before in 2 ml of cold 25% (wt/wt) sucrose–5 mM EDTA, pH 7.5.

One milliliter of the membrane suspension was layered on top of sucrose step gradients prepared as described previ-



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                1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21 22  23 24 25 26 27 28 29 30
pRSG192  MetLeuLysHisSerLeuIleAlaAlaSerValIleThrThrLeuAlaGlyCysSerSerLeuGln/SerSerGluGlnGlnValValAsn

pRSG196  MetThrMetIleThrProSerLeuHisAlaCysArgSerThrLeuGluAspProArgValPro/SerSerGluGlnGlnValValAsn
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FIG. 1. Genetic and physical maps of plasmids containing the *V. harveyi chb* gene. (A) Restriction and genetic maps of pRSG14, pRSG16, pRSG192, and pRSG196. The solid bars represent cloned *V. harveyi* sequences. The chitobiase gene coding region (*chb*) is indicated. Restriction sites: A, *Ava*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sau*3A; Sa, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*I; X, *Xba*I. (B) Amino acid sequence of the amino terminus of prechitobiase, based on DNA sequence and open reading frame analysis (to be published elsewhere), showing the translational fusion created in pRSG196 by deletion of an *Sst*I fragment from pRSG192. The slashed line indicates where the fusion occurred.

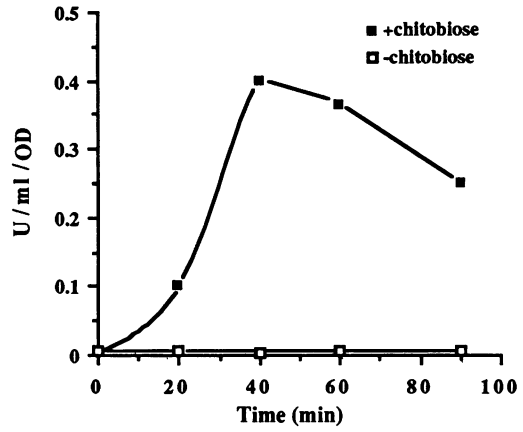


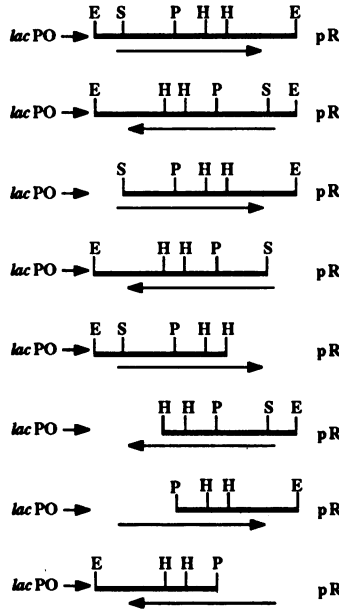
FIG. 2. Induction of *V. harveyi* chitobiase activity with chitobiose. *V. harveyi* was grown in LM broth to an OD_{450} of 0.2. The culture was divided, and half was incubated in the presence of 0.2 mM chitobiose (■), the other half in the absence of chitobiose (□). Chitobiase activity and cell growth were followed for 90 min from the time that chitobiose was added. Chitobiase activity was measured by method 1 with PNAG as a substrate and is expressed as units of activity per milliliter of cell culture per OD_{450} unit of the culture.

ously (20), and the gradients were centrifuged to equilibrium at 35,000 rpm in a Beckman SW40 Ti rotor for 15 h at 4°C. The gradients were fractionated by collecting 200-μl fractions from the top.

RESULTS

Characterization of the chitobiase gene *chb*. The *V. harveyi* B392 genomic bank, prepared by cloning a *Sau*3A partial digest of *V. harveyi* DNA into the single *Bam*HI site within the *tet* gene of pMK2004, contained two clones with chitobiase activity (*chb*) and one clone with an *N*-acetyl-D-glucosaminidase lacking chitobiase activity (24). The three clones were detected by the appearance of the yellow *p*-nitrophenylate product after individual colonies of the 2,450-member clone bank were sprayed with 10 mM PNAG dissolved in 100 mM sodium phosphate, pH 7.0. The restriction maps of the two plasmids encoding chitobiase activity, pRSG14 and pRSG16, from this clone bank are shown in Fig. 1. The insert sizes for each were 5.3 and 13.5 kilobases (kb), respectively. To examine the possibility that *chb* regulatory elements might also be included in the cloned DNA, induction studies with chitobiose were carried out with *E. coli* cells containing the cloned *chb* gene and with *V. harveyi* cells. Chitobiase activity, induced by chitobiose in *V. harveyi*, was detected within 20 min after the addition of chitobiose to exponentially growing *V. harveyi* cells and reached a maximum level between 30 and 50 min after induction (Fig. 2). The activity of chitobiase then diminished to approximately 62.5% of the maximal level within 90 min after induction. With the *E. coli* clones, however, there was no significant change in the activity of chitobiase after the addition of chitobiose to the growth medium, and the levels of activity in the absence of chitobiose were similar (pRSG16) or higher (pRSG14) than those found for *V. harveyi* induced with chitobiose (Table 1). The higher level of chitobiase activity seen with pRSG14 compared with pRSG16 was probably due to additional transcription from the *tet* promoter in pRSG14.

Subcloning and deletion analysis of the insert in pRSG14 (Fig. 3) was performed with the cloning vector pUC19, which contained the *E. coli lac* promoter-operator region and the α fragment of the *lacZ* gene (26). The chitobiase gene was subcloned and located to within a 3.5-kb *Eco*RI fragment of pRSG14 (pRSG192 and pRSG191, Fig. 3). Both plasmids showed high levels of chitobiase, equivalent to induced levels in *V. harveyi*. When the inducer IPTG was added, increased expression was noted in only one orientation, indicating the direction of transcription of the *chb* gene



Plasmid	Chitobiase activity − IPTG	Chitobiase activity + IPTG
pRSG192	9.90	22.10
pRSG191	1.23	1.17
pRSG196	1.24	4.42
pRSG186	<0.06	<0.06
pRSG184	<0.06	<0.06
pRSG194	<0.06	<0.06
pRSG193	<0.06	<0.06
pRSG183	<0.06	<0.06

FIG. 3. Chitobiase activity associated with plasmids containing restriction fragments from the *V. harveyi chb* region. S, *Sst*I; other abbreviations are the same as in Fig. 1. Arrows indicate the direction of transcription and, if under the insert, the size of the chitobiase (*chb*) gene. The *chb* gene was subcloned in a 3.5-kb *Eco*RI fragment from pRSG14 into the *Eco*RI site of pUC19 in both orientations (pRSG191 and pRSG192). Plasmid pRSG196 is the result of deleting a 0.5-kb *Sst*I fragment from pRSG192, and pRSG186 (pUC18 derivative) is a similar *Sst*I deletion of pRSG182. pRSG182 (not shown) contains the 3.5-kb *Eco*RI fragment inserted into the *Eco*RI site of pUC18 (26) in the same orientation with regard to the *lac* promoter as pRSG191. The polylinker in pUC18 is in the opposite orientation from that in pUC19. Plasmids pRSG192 and pRSG184 were constructed by deleting *Hin*dIII fragments from pRSG191 and pRSG182, respectively. Plasmids pRSG193 and pRSG183 were constructed by deleting a *Pst*I fragment from pRSG192 and pRSG181, respectively. *E. coli* JM109 cells harboring the plasmids described above were grown to an OD_{450} of 0.4. IPTG (0.1 mM) was added to half of the culture (+ IPTG), and incubation was continued for an additional 1.5 h. Chloramphenicol (200 μg/ml) was added, and the OD_{450} was measured. The cells were centrifuged and washed once with M9 salts. Cells containing plasmids pRSG191 and pRSG192 were treated with Sarkosyl as described in Materials and Methods. Cells harboring other plasmids were not treated with Sarkosyl; instead, they were concentrated 10-fold in 10 mM Tris chloride, pH 7.3, and sonicated for five 40-s pulsed intervals at an output of 5 and a 50% duty cycle with a microtip attached to a Bronson cell disruptor 350. Chitobiase activity was determined by Method 1 with PNAG as the substrate and is in units per milliliter of culture per OD_{450} unit. *lac*PO, *lac* promoter-operator.

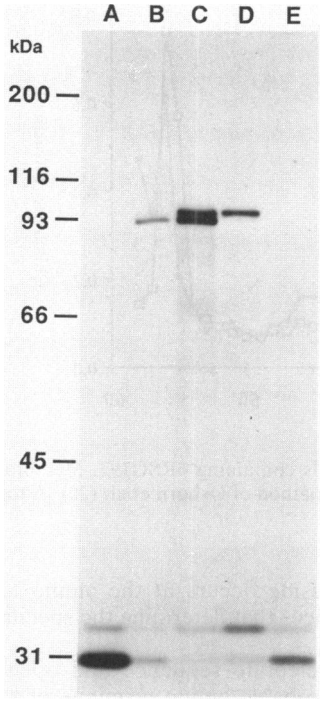


FIG. 4. Autoradiograms of ^{35}S-labeled polypeptides encoded by plasmids pMK2004 (A), pRSG14 (B), pRSG192 (C), pRSG196 (D), and pUC19 (E). Protein standards (left) were from Bio-Rad.

shown by the arrow in Fig. 3. The length of the arrow corresponds to the size of the *chb* gene as determined from minicell analysis of plasmid-encoded proteins (see below) and open reading frame analysis of the nucleotide sequence (to be published elsewhere). Plasmid pRSG196 (Fig. 3) has a deletion of a small 0.5-kb *Sst*I fragment next to the *lac* promoter region in pRSG192 (Fig. 1). Chitobiase activity associated with this plasmid was only detected after sonication because the enzyme was not transported to the outer membrane, as shown below. Here again, induction with IPTG occurred in only one orientation, and the lack of activity in the other orientation (pRSG186), whether or not IPTG was added, indicated that a sequence with promoter activity had been deleted. The increased levels of activity observed in the absence of IPTG with pRSG192 compared with pRSG191 and with pRSG196 compared with pRSG186 indicate that the *lac* promoter was not completely repressed by the concentration of *lac* repressor present in strain JM109. The lack of activity associated with the smaller fragments cloned in plasmids pRSG181, pRSG184, pRSG193, and pRSG194 delimits the *chb* gene with its presumptive promoter to the 3.5-kb *Eco*RI fragment.

By knowing the amino acid sequence of prechitobiase, which was deduced from the nucleotide sequence of the *chb* gene (to be published elsewhere), and knowing the sequence of pUC19 (26), it was apparent that pRSG196 contained a translational fusion by which 22 amino acids from the amino terminus of prechitobiase were replaced with 21 amino acids from the LacZ(α) amino terminus of pUC19 (Fig. 1B). There is significant homology centered at amino acids 15 to 19 in the amino terminus of prechitobiase (Fig. 1B) to six amino acids (Leu-Ala-Gly-Cys-Ser-Ser) at the processing and modification site of the major outer membrane lipoprotein (2, 17).

Minicell analysis of proteins encoded by plasmids containing the *chb* gene. Figure 4 shows the electrophoretic pattern of ^{35}S-labeled proteins synthesized in minicells of *E. coli* p678-54 carrying the hybrid plasmids pRSG14, pRSG192, and pRSG196. The two plasmids, pRSG14 and pRSG192, which carry the complete *chb* gene gave rise to a polypeptide that was 92.1 kDa in size (Fig. 4, lanes B and C) in addition to the polypeptides encoded by the vectors (lanes A and E). A larger polypeptide of 95.3 kDa also appeared with pRSG192 (lane C), and since the insert DNA in this plasmid contains only enough coding space for one protein of this size, the larger band must correspond to the unprocessed form of the enzyme. With pRSG196, which codes for the fusion protein (Fig. 1), only a larger unprocessed form of the protein was found (Fig. 4, lane D).

Cellular location of chitobiase in *E. coli* cells containing the *chb* gene. *E. coli* cells containing either pRSG192 or pRSG196 (Fig. 1) were grown in low-phosphate medium containing IPTG. The combination of low phosphate and IPTG led to increased synthesis in these cells of alkaline phosphatase, β-galactosidase, and chitobiase. The cells were fractionated into extracellular, periplasmic, and cytoplasmic plus membrane fractions as described in Materials and Methods, and the fractions were assayed for chitobiase; β-galactosidase, a cytoplasmic enzyme; and alkaline phosphatase, a periplasmic enzyme (Table 2). Most of the chitobiase activity exhibited by either pRSG192 or pRSG196 was found in the cytoplasmic plus membrane fraction. When this fraction was separated into cytoplasmic and membrane fractions by high-speed centrifugation, however, very different results for the two plasmids were found (Table 2). Two-thirds of the chitobiase activity associated with pRSG192 was recovered in the membrane fraction, while 99% of the activity associated with pRSG196 was found in the cytoplasmic fraction. The fusion protein, although active, was apparently not transported to the membrane, implicating the involvement of the amino terminus region of chitobiase in this translocation event.

Chitobiase in the outer membrane of *E. coli* cells. The membranes of *E. coli* cells were separated by sucrose gradient centrifugation by the method of Osborn et al. (20) to determine whether the membrane-associated chitobiase was in the inner or outer membrane (Fig. 5). Four membrane bands were obtained, similar to previously described results (20). Bands L1 and L2 were previously characterized as cytoplasmic membrane, H as outer membrane, and the minor band, M, as unseparated envelope fragments (20). Only one peak of chitobiase activity was resolved by this method, which corresponded to the outer membrane band (H) near the bottom of the gradient. Chitobiase was loosely associated with intact *E. coli* cells containing the *chb* gene; up to 50% of the activity could be solubilized with sonication (data not shown) or detergent (Soto-Gil et al., in press), possibly accounting for the chitobiase activity observed at the top of the gradient (Fig. 5) and in the cytoplasmic fraction (Table 2).

In *V. harveyi*, chitobiase activity also appeared to be associated with the membrane. *V. harveyi* cells grown at 30°C and induced for 1 h with 0.2 mM chitobiose were lysed and treated as described in Materials and Methods for separation of membranes into inner and outer fractions. The chitobiase activity recovered in the membrane fraction was 68.5%, that in the culture supernatant was 7.9%, and that in the soluble cell fraction was 23.6%. It was not possible to associate chitobiase activity with a particular membrane for *V. harveyi* because attempts to separate the inner and outer

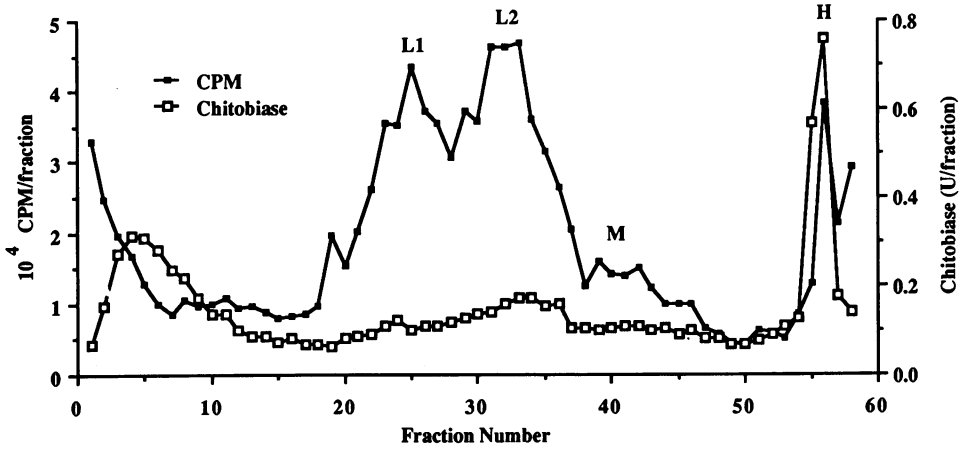


FIG. 5. Sucrose density gradient fractionation of membranes isolated from *E. coli* cells containing pRSG192. Membrane phospholipids were labeled with [^{3}H]glycerol, and the membranes were isolated and fractionated by the method of Osborn et al. (20). A total of 60 fractions (200 μl/fraction) were collected, with fraction 1 coming from the top of the gradient.

membranes of *V. harveyi* with Osborn gradients were unsuccessful.

DISCUSSION

Two plasmids encoding chitobiase activity were isolated from a genomic bank of *V. harveyi* DNA constructed in *E. coli*. The plasmids each contained a 3.5-kb *Eco*RI fragment that when subcloned conferred chitobiase activity. Minicell analysis of plasmid-encoded proteins and deletion analysis of this *Eco*RI fragment established that the chitobiase gene (*chb*) coded for a single polypeptide with a molecular mass of 95.3 kDa, and this protein was processed to a smaller polypeptide of 92.1 kDa. The transcription orientation of the *chb* gene was determined by using transcription fusions between the *lac* promoter and the *chb* gene. Promoter activity was associated with the sequence directly upstream of the *chb* gene. It remains to be established that this is a promoter used by *V. harveyi*. Although *V. harveyi* and *E. coli* are in different families, they are similar enough that the *V. harveyi* chromosomal origin of replication functions in *E. coli* (27), so promoter recognition may not have diverged significantly between the two organisms.

Fractionation studies of *E. coli* cells containing the *chb* gene in pRSG192 (Fig. 1), in which cells were separated into membrane, cytoplasmic, and periplasmic fractions, located the chitobiase activity to the membrane fraction. Similar results were obtained with *V. harveyi* cells induced with chitobiose. When the *E. coli* membranes were further separated with an Osborn gradient (20) into inner and outer membrane fractions, the major portion of the chitobiase activity was recovered in the outer membrane fraction. In similar studies performed with *E. coli* cells containing pRSG196, which is missing the 0.5-kb *Sst*I fragment of pRSG192 (Fig. 1), 98% of the chitobiase activity was located in the cytoplasm. From the nucleotide sequences of the *chb* gene and the cloning vector, pUC19, it was possible to determine that the *Sst*I deletion used to produce pRSG196 created an in-frame fusion of the amino terminus of the *lacZ*(α) gene from pUC19, replacing the first 22 amino acids of prechitobiase. The minicell results and cell fractionation studies with pRSG192 and pRSG196 indicate that prechitobiase is processed by removal of a signal peptide, probably during transport to the outer membrane of *E. coli*, and that processing occurs at the amino terminus, which contains sequences that determine the specificity of processing.

Based on nucleotide sequence and open reading frame analyses, the probable amino terminus of the unprocessed enzyme (to be published elsewhere) was found to contain a homology to the outer membrane lipoprotein. Currently, we are examining whether the six amino acids identical to the amino acids adjacent to the processing and modification site in the outer membrane lipoprotein of *E. coli* is the site where processing of prechitobiase occurs and whether chitobiase is, in fact, a lipoprotein.

There is no evidence yet indicating that the genes encoding chitinase and chitobiase activities are linked in *V. harveyi*. We have isolated two different chitinase genes from *V. harveyi* genomic banks, and these clones do not exhibit chitobiase activity (to be published elsewhere). Also, cells containing the *chb* plasmids pRSG14 and pRSG16 (Fig. 1) did not exhibit chitinase activity; the activity previously attributed to these plasmids was due to the lysozyme used to prepare cell extracts (24). The chitinase and chitobiase genes therefore appear to be transcribed from separate promoters, and since the product of one enzyme is the substrate of the other, expression from these promoters may not be coordinately controlled.

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LITERATURE CITED

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening plasmid DNA. Nucleic Acids Res. 7:1513–1523.
2. **Braun, V.** 1975. Covalent lipoprotein from the outer membrane of *Escherichia coli*. Biochim. Biophys. Acta **415**:335–377.
3. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl.

Acad. Sci. USA **62**:1159–1166.
4. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69**:2110–2114.
5. **Fuchs, R. L., S. A. McPherson, and D. J. Drahos.** 1986. Cloning of a *Serratia marcescens* gene encoding chitinase. Appl. Environ. Microbiol. **51**:504–509.
6. **Fujimura, F. K., J. W. Zyskind, and D. W. Smith.** 1978. The *Escherichia coli dnaB* protein is required for initiation of chromosomal DNA replication. Cold Spring Harbor Symp. Quant. Biol. **43**:559–562.
7. **Garen, A., and C. Levinthal.** 1960. A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. Purification and characterization of alkaline phosphatase. Biochim. Biophys. Acta **38**:470–483.
8. **Horwitz, M., J. Reid, and D. Ogrydziak.** 1984. Genetic improvement of chitinase production by *Serratia marcescens*, p. 191–208. *In* J. P. Zikakais (ed.), Chitin, chitosan, and related enzymes. Academic Press, Inc., New York.
9. **Jeuniaux, C.** 1978. Distribution and quantitative importance of chitin in animals, p. 5–10. *In* R. A. A. Muzzarelli (ed.), Proceedings of the First International Conference on Chitin/Chitosan. MIT Sea Grant, Boston.
10. **Jones, J. D. G., K. L. Grady, T. V. Suslow, and J. R. Bedrock.** 1986. Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens*. EMBO J. **5**:467–473.
11. **Kahn, M., R. Kolter, C. Thomas, D. Figurski, K. Meyer, E. Remaut, and D. Helinski.** 1979. Plasmid cloning vehicles derived from plasmids ColE1, F, R6K, and RK2. Methods Enzymol. **68**:268–280.
12. **Koshland, D., and D. Botstein.** 1980. Secretion of beta-lactamase requires the carboxy end of the protein. Cell **20**:749–760.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Matsumura, P., M. Silverman, and M. Simon.** 1977. Synthesis of *mot* and *che* gene products of *Escherichia coli* programmed by hybrid ColE1 plasmids in minicells. J. Bacteriol. **132**:996–1002.
15. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Monreal, J., and E. Reese.** 1969. The chitinase of *Serratia marcescens*. Can. J. Microbiol. **15**:689–696.
17. **Nakamura, K., and M. Inouye.** 1979. DNA sequence of the gene for the outer membrane lipoprotein of *E. coli*: an extremely AT-rich promoter. Cell **18**:1109–1117.
18. **Nealson, K. H., and J. W. Hastings.** 1977. Bacterial luminescence: its control and ecological significance. Microbiol. Rev. **43**:496–518.
19. **Ohtakara, A., M. Mitsutomi, and Y. Uchida.** 1979. Purification and some properties of chitinase from *Vibrio* sp. J. Ferment. Technol. **57**:169–177.
20. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonela typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962–3972.
21. **Reichelt, J. L., P. Baumann, and L. Baumann.** 1976. Study of genetic relationships among marine species of the genera *Beneckea* and *Photobacterium* by means of in vitro DNA/DNA hybridization. Arch. Microbiol. **1**:101–120.
22. **Reissig, J. L., J. L. Strominger, and L. F. Leloir.** 1955. A modified colorimetric method for the estimation of *N*-acetylamino sugars. J. Biol. Chem. **217**:959–966.
23. **Ruiz-Herrera, J.** 1978. The distribution and quantitative importance of chitin in fungi, p. 11–21. *In* R. A. A. Muzzarelli (ed.), Proceedings of the First International Conference on Chitin/Chitosan. MIT Sea Grant, Boston.
24. **Soto-Gil, R. W., and J. W. Zyskind.** 1984. Cloning of *Vibrio harveyi* chitinase and chitobiase genes in *Escherichia coli*, p. 209–223. *In* J. P. Zikakis (ed.), Chitin, chitosan, and related enzymes. Academic Press, Inc., New York.
25. **Wortman, A. T., C. C. Somerville, and R. R. Colwell.** 1986. Chitinase determinants of *Vibrio vulnificus*: gene cloning and applications of a chitinase probe. Appl. Environ. Microbiol. **52**:142–145.
26. **Yanisch-Perron, C., J. Vierria, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
27. **Zyskind, J. W., J. M. Cleary, W. S. A. Brusilow, N. E. Harding, and D. W. Smith.** 1983. Chromosomal replication origin from the marine bacterium *Vibrio harveyi* functions in *Escherichia coli*: *oriC* consensus sequence. Proc. Natl. Acad. Sci. USA **80**:1164–1168.