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Cloning and Expression in *Escherichia coli* of the Gene for Extracellular Phospholipase A1 from *Serratia liquefaciens*

MICHAEL GIVSKOV,* LARS OLSEN, AND SØREN MOLIN

Department of Microbiology, The Technical University of Denmark, DK-2800 Lyngby, Denmark

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From a genomic library of *Serratia liquefaciens*, a cloned DNA fragment comprising a two-gene operon was isolated and expressed in *Escherichia coli*. One of the gene products was identified as a phospholipase A1, and the enzyme was found to be excreted to the outer environment from *S. liquefaciens* as well as from *E. coli*. Both genes were sequenced, and the relationship between open reading frames in the DNA sequence and in vitro-expressed polypeptides was established. The length of the phospholipase polypeptide was found to be 319 amino acids. In the amino-terminal end of the coding sequence was a stretch of about 20 hydrophobic amino acids, but, in contrast to consensus signal peptides, no basic residues were present. The length of the second polypeptide was 227 amino acids. It was found that expression of the phospholipase gene in both *E. coli* and *S. liquefaciens* was growth phase regulated (late expression).

Many aspects of the mechanism of protein transport have been elucidated through studies of eucaryotic model systems, but it has been found that several of these characteristics are shared by procaryotes; in fact, there are examples showing that extracellular proteins from higher cells are also exported from bacteria after gene cloning (18). Among the key features of the transport mechanism is the presence of hydrophobic signal peptides in the proteins and localization in the membrane of peptidases that remove the signals as the proteins pass through (16). There are also indications of a coupling between translation and transport, although in bacteria the evidence is circumstantial (14).

The group of gram-negative bacteria is special in the sense that the cells are surrounded by two membranes. In most cases the outer membrane is an efficient barrier against total export of proteins, and hence most model systems studied in these bacteria (mainly *Escherichia coli*) involve proteins whose destination is either the periplasmic space between the two membranes or the outer membrane itself. In these cases the requirement for signal peptides is well established (16), and several mutants defective in various parts of the transport apparatus have been isolated and characterized (8).

There are, however, a number of gram-negative bacteria which possess the capacity to totally excrete certain proteins to the outside environment. Among these we have chosen two species of the genus *Serratia* as model organisms. There are two major reasons for this choice. (i) *Serratia* spp. belong to the family *Enterobacteriaceae*, to which *E. coli* also belongs, and it is possible to exploit most, if not all, of the recombinant DNA tools developed for *E. coli* for use with this group of organisms. (ii) *Serratia* spp. are known to export a number of hydrolytic enzymes (nuclease, lipases, proteases, and chitinase) to the growth medium. Our aim is to understand the basis for this capacity to translocate proteins across two membranes, and our main approach at present is to use genetics. We have cloned the genes encoding most of these exoenzymes from *Serratia marcescens* and *Serratia liquefaciens* into *E. coli*, and in this communication a first analysis of the phospholipase gene from *S. liquefaciens* is described.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains. Strains used were *E. coli* JM103 [Δ(*lac-pro*) *thi rpsL supE endA sbcB15 hsdR4* (F′ *traD36 proAB lacI-ZΔ15*)] (7), *E. coli* CSH50 [Δ(*lac-pro*) *rpsL thi* (9), *E. coli* MC1000 [*araD139* (*ara-leu*) *7697* Δ*lac thi*] (laboratory stock), *E. coli* MT102 (*hsdR* derivative of MC1000) (laboratory stock), *E. coli* 1100 (*endA thi*) (laboratory stock), *S. marcescens* W225 (20), and *S. liquefaciens* (our own isolate).

Media and growth conditions. Bacterial cells were grown in LB or phosphate-buffered minimal medium supplemented with Casamino Acids (Difco Laboratories, Detroit, Mich.) and thiamine. Growth was followed spectrophotometrically at an optical density of 450 nm (OD_{450}). Solid media contained LB or minimal medium supplemented with Casamino Acids and thiamine. Antibiotics were dissolved in the media at the following concentrations: ampicillin, chloramphenicol, and kanamycin, 50 μg/ml; and tetracycline, 8 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was used in plates at a concentration of 50 μg/ml. Solid media for detection of phospholipase activity contained 2 ml of egg yolk mixed with 2 ml of 0.9% NaCl per 20 ml of LB agar, minimal medium supplemented with 1% peptone and 2% skim milk (for protease activity), and DNase test agar (Difco) (for nuclease activity). Lipase activity was screened in liquid cultures with the chromophoric substrate *para*-nitrophenyl palmitate as described elsewhere (20).

Measurements of enzyme activity. Activities of β-galactosidase were measured essentially according to Miller (9). Activities of β-lactamase were measured by using the chromophoric substrate nitrocefin as described by the manufacturer (BBL Microbiology Systems, Cockeysville, Md.). The reaction was stopped by addition of 0.4 M guanidine hydrochloride. Phospholipase activity was measured in 1% agarose gels containing 0.1 volume of egg yolk in 0.5% NaCl–0.2% sodium dodecyl sulfate (SDS) and 0.1 M Tris (pH 11)–0.9% NaCl. The activity was measured as diffusion speed of the white precipitating zone in the gel and calculated as change in precipitation radius per unit of time (millimeters per hour). A linear relationship was found between the logarithm of the enzyme concentration (a serial dilution of concentrated enzyme sample) and the diffusion speed. Enzyme activities were expressed as fractions of a concentrated sample.

Cell fractionation. Cells were fractionated essentially as described elsewhere (12) with the following modifications. Samples (1 ml) were centrifuged twice, and the cell-free supernatants were saved. The cell pellets were suspended in 0.1 ml of 30 mM Tris (pH 8.0)–20% (vol/wt) sucrose and treated with 7 μg of lysozyme per ml and 10 mM EDTA for 25 min on ice. After 5 min of centrifugation, the supernatant was used as the membrane-free periplasmic fraction, and the spheroplasts in the pellet were osmotically lysed upon suspension in 30 mM Tris (pH 8.0).

Isolation of total DNA. LB cultures of 150 ml were grown overnight to high density. The cells were harvested and washed twice in TES buffer (10 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA), resuspended in 20 ml of TES buffer containing 1 mg of lysozyme per ml, and incubated at 37°C for 30 min. Cells were lysed by adding 1% SDS and further incubated for 1 h. Cellular debris was removed by centrifugation, and the supernatant was extracted twice with phenol at 37°C. The supernatant was then carefully mixed with isopropanol containing 0.3 M sodium acetate. Long threads of DNA were recovered with a bended glass spatula, washed several times in 80% ethanol, and suspended in TE buffer (10 mM Tris [pH 7.4]–1 mM EDTA). This DNA was used as a source for construction of a genomic library in *E. coli*.

Purification of plasmid DNA. Plasmid DNA was prepared essentially as described by Maniatis et al. (5). Cesium chloride gradient centrifugations were used to purify plasmid DNA (17). DNA restriction fragments and plasmid DNA were purified from agarose gels by electroelution in 0.5× TBE buffer (45 mM Tris borate, 45 mM boric acid, 1 mM EDTA), phenol extracted twice, ethanol precipitated, and ethanol washed.

DNA sequencing. DNA was sequenced by the dideoxy-chain termination method developed by Sanger et al. (15), using [^{35}S]dATP the Klenow fragment of DNA polymerase I, and bacteriophage M13 derivatives mp8 and mp9. All reactions were initiated from the universal sequencing primer. All chemicals were obtained from Amersham Corp. (Arlington Heights, Ill.). Methods were followed according to the protocol of Amersham, with changes in the dideoxynucleotide triphosphate concentrations that would match the GC-rich *Serratia* DNA. Deazo-dGTP (Boeringer Mannheim Biochemicals, Indianapolis, Ind.) was used in the sequencing reactions. Purified *Eco*RI fragment of plasmid pMG200 was digested with *Taq*I, *Sau*3A, *Alu*I, *Cla*I, *Pst*I, *Eco*RV, or *Bst*EII and shotgun cloned into mp8 or mp9. The resulting sequences were merged and oriented with respect to cloned and sequenced *Sal*I-*Eco*RI, *Sma*I-*Eco*RI, and *Sma*I-*Sal*I fragments (see Fig. 1).

S1 nuclease mapping. From cells grown in AB minimal medium with Casamino Acids, RNA was extracted with phenol (three times at 60°C). The 200-base-pair (bp) *Eco*RI-*Hin*dIII fragment from pMG230 was 5′ end labeled with ^{32}P, digested with *Bam*HI, and used as a hybridization probe. In each hybridization reaction, 200 μg of total RNA and 0.2 μg of labeled probe were used. The reaction mixtures were denatured at 80°C for 10 min in 80% formamide–0.4 M NaCl–5 mM EDTA–30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid) (pH 6.5) in a volume of 130 μl and allowed to hybridize at 45°C overnight. Unhybridized DNA was digested with 100 U of S1 nuclease in 0.4 M NaCl–1 mM ZnCl–5% glycerol–30 mM sodium acetate (pH 4.2) in a volume of 400 μl. Samples were taken 3, 6, 12, and 24 min after nuclease addition, phenolized, ethanol precipitated, dissolved in 8 M urea, and analyzed by electrophoresis on 8% polyacrylamide sequencing gels containing 8 M urea. The purine-specific reaction of Maxam and Gilbert (6) was performed on the hybridization probe, coelectrophoresed with the samples, and used as a marker.

DNA hybridization. DNA binding to nitrocellulose paper (Gene-Screen Plus; Dupont, NEN Research Products, Boston, Mass.) and hybridization were performed essentially as described by the manufacturer. Nick translation of DNA according to the protocol of Amersham was performed by using the isotope [^{35}S]dATP and DNA polymerase I.

In vitro transcription-translation. Cell extracts of exponentially growing *E. coli* cells were obtained from Amersham and as a gift from P. V. Hansen. The DNA templates used were purified from agarose gels by electroelution. In vitro transcription-translation was performed according to Zubay (21). Polypeptides expressed in this system were radioactively labeled with [^{35}S]methionine and analyzed on SDS-polyacrylamide gels.

Construction of expression vector pMG300. Plasmid DNA of pHC624 (1) was restricted with *Bgl*II and ligated to *Bgl*II-restricted lambda *c*I857 DNA. Colonies resistant to phage lambda were isolated, DNA was purified from resistant colonies, and the orientation of the lambda *Bgl*II fragment was determined (Fig. 1). DNA inserted in the unique restriction sites *Sal*I, *Bam*HI, *Sma*I, and *Eco*RI will be transcribed from the rightward lambda promoter p_R. Also, construction of protein fusions is a possibility because of the presence of the 28 amino-terminal codons of the lambda *cro* gene upstream of the cloning sites.

Blotting of phospholipase activity. The position of phospholipase in an SDS-polyacrylamide gel was visualized in the following way. The protein samples were heat denatured at 90°C in the presence of SDS and β-mercaptoethanol, loaded onto the gel, and electrophoresed. The gel was then allowed to swell in 30 mM Tris (pH 8.0)–0.9% NaCl for 30 min. Excess buffer was removed, and a suspension of warm 1% agarose-egg yolk in 0.1 M Tris (pH 11)–0.9% NaCl was layered on top of the gel and allowed to harden. On the next day, the position of phospholipase activity was observed as a dense white precipitation zone in the agarose-egg yolk gel. Either the gel was photographed or the position of the precipitation was marked on transparent overhead sheets, and the acrylamide gel was stained, dried, and autoradiographed.

RESULTS

Characterization of the phospholipase expressed in *S. liquefaciens*. The strain of *S. liquefaciens* used was isolated by us from liquefied plant tissue (cucumber) and later identified as *S. liquefaciens*. This species is closely related to the better-known *S. marcescens*, and as far as excretion of hydrolytic enzymes is concerned, there seem to be very few, if any, differences between these two bacterial species. We have so far concentrated on the following exoenzymatic activities of *S. liquefaciens*: nuclease, lipase, protease, and phospholipase.

Phospholipase activity was easily observed on plates containing egg yolk, on which positive clones developed turbid precipitation zones as haloes around the colonies (data not shown). Similar reactions could be obtained if cell-free growth medium from an outgrown culture of *S. liquefaciens* was spotted onto these indicator plates, indicating total excretion of the enzyme. The egg yolk assay was used quantitatively, as described in Materials and Methods, since it was found that there was a simple relationship between activity and the rate of diffusion of the precipitation

FIG. 1. Construction of plasmids. pMG200 carries a 3.2-kbp *Eco*RI fragment obtained from *S. liquefaciens* inserted in the *c*I repressor gene of pNU121. All other plasmids except pMG300 (construction of which is described in Materials and Methods) are derived from this hybrid. Solid blocks indicate the presence of *Serratia* DNA; slanted lines indicate deletions of restriction enzyme fragments. Plasmid phenotypes are shown as + (phospholipase producing) and − (non-phospholipase producing). Clones carrying pMG300 derivatives were tested at 30 and 42°C for phospholipase production. In pMG230, a 200-bp *Bam*HI-*Pst*I obtained from pMG329 was cloned into the translational fusion vector pNM481 (10), resulting in an in-frame fusion between *phlA* and *lacZ*. Enzyme abbreviations: B1, *Bam*HI; B2, *Bgl*II; E1, *Eco*RI; E5, *Eco*R5; F, *Fsp*I; P, *Pst*I; S, *Sma*I; S1, *Sal*I; S2, *Sac*II.

zone. Thus, phospholipase activity was measured throughout this analysis by taking samples of bacterial cultures from growth medium and applying them to agarose-egg yolk gels, followed by incubation for development of precipitation zones. Strains of both *S. liquefaciens* and *S. marcescens* appeared positive after 16 to 30 h when plated on the egg yolk indicator plates, whereas *E. coli* showed no sign of extracellular phospholipase activity even after several days of incubation (not shown).

In Fig. 2 is shown what seems to be characteristic of many bacterial exoenzymes: growth-phase-dependent expression of phospholipase from *S. liquefaciens*. A very low rate of enzyme synthesis was observed in the exponential growth phase, but when growth began to slow down (the transition between exponential growth and stationary phase), there was a sudden burst of expression which eventually came to a halt when the cells stopped growing. The problem of this late expression is dealt with in more detail in a separate communication (submitted for publication).

Construction of a genomic library in *E. coli*. Total *S. liquefaciens* DNA was restricted with *Eco*RI, and fragments were cloned into the *Eco*RI site of the vector pNU121 (11). This cloning vector allows direct selection for chimeric plasmid molecules, since several unique restriction sites (e.g., *Eco*RI) are located in the *c*I gene, whose product represses the expression of the *tet* gene. Thus, tetracycline-resistant transformants will appear as a consequence of insertion of DNA fragments in these sites. Upon transformation to the host restriction-negative strain of *E. coli* K-12, MT102, cells receiving hybrid plasmids were selected on tetracycline-containing plates. The total gene bank contained approximately 40,000 colonies with a background (i.e., colonies carrying nonhybrid plasmids) of less than 1%. The genomic library was screened for phospholipase, nuclease, lipase, and protease exoenzyme activities (see Materials and Methods).

In screening 4,000 colonies, we isolated 15 phospholipase-producing colonies, 2 nuclease-producing colonies, and 1 lipase-producing colony. None expressed more than one of the tested enzymatic activities. None of the 15 phospholipase-positive clones reacted with *para*-nitrophenyl palmitate, nor did the lipase-producing clone react positively on egg yolk plates. *E. coli* colonies were negative for all exoenzyme activities.

Only one phospholipase-producing clone, pMG200, was analyzed in detail. This clone contained a plasmid with a single 3.2-kbp *Eco*RI fragment inserted in the *c*I gene of pNU121. The fragment hybridized to total *S. liquefaciens* and *S. marcescens* DNA but not to total *E. coli* DNA (not shown). In a separate analysis consisting of enzymatic degradation of two position-specific phospholipids, separation of the reaction products by thin-layer chromatography, and identification of these products by gas chromatography, we found that cell-free supernatants prepared from *E. coli* harboring pMG200 (in contrast to purified snake venom phospholipase A2 and control supernatants from *E. coli* harboring pNU121) preferentially liberated the fatty acid in position 1 of the phospholipids (not shown). Therefore, we conclude that the enzyme responsible for the positive reaction on the egg yolk indicator plates was a phospholipase A1.

Excretion of phospholipase from an *E. coli phl*$^+$ clone. Isolation of several clones of *E. coli* from the genomic library of *S. liquefaciens* DNA which were positive on egg yolk plates indicated that even in *E. coli* the *Serratia* phospholipase is extracellular. However, these simple plate tests do not distinguish between partial leakage from lysed cells and true specific excretion. We therefore performed a more extensive analysis of the compartmentalization of phospholipase in both *S. liquefaciens* and *E. coli*. The extent of liberation of enzyme activity from the cells was quantified through fractionation of the cells (*S. liquefaciens* and *E. coli* harboring pMG200), and the activity of phospholipase was determined in the cell-free supernatant, the periplasmic fraction, and the spheroplasts. Similar measurements of *E. coli* β-lactamase (periplasmic enzyme) and β-galactosidase (cytoplasmic enzyme) activities were performed to monitor cell lysis and the efficiency of cell fractionation. As expected, β-galactosidase activity was found primarily in the cytoplasmic fraction, whereas β-lactamase activity was located in the periplasm (Table 1). This distribution was the

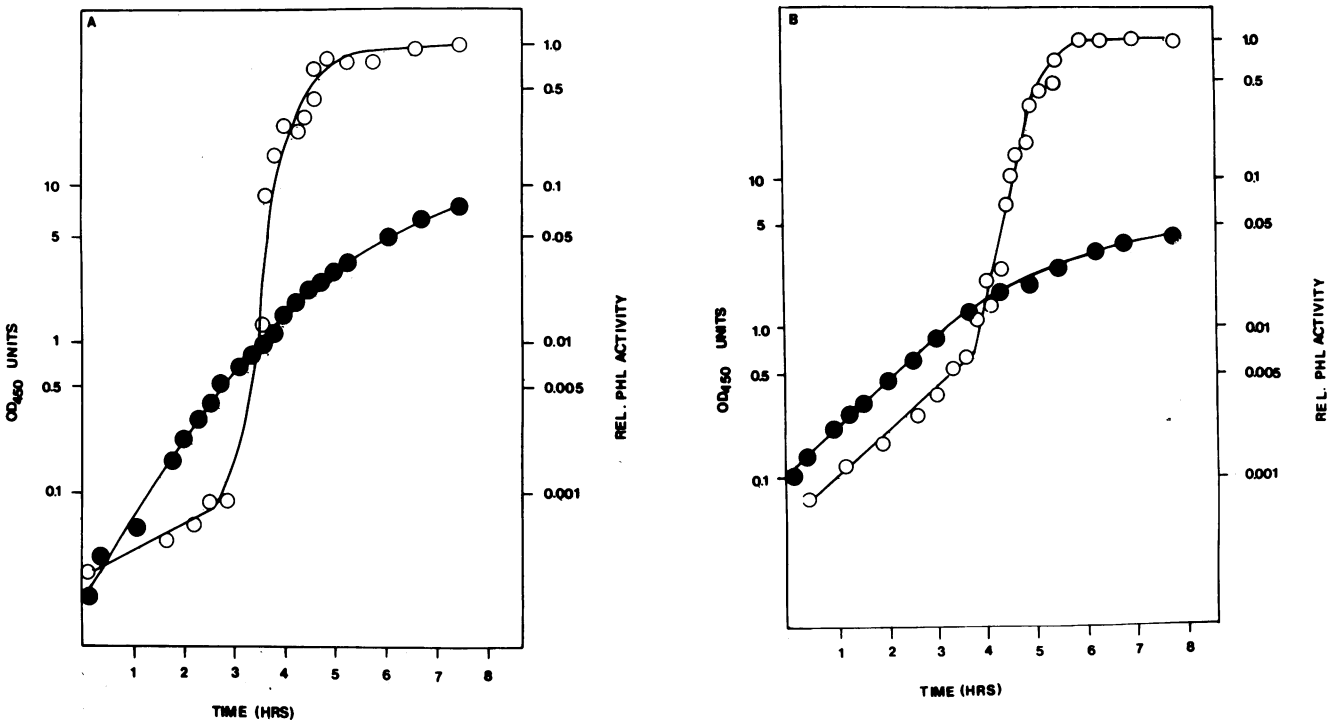

FIG. 2. Relationship between growth of bacteria and production of phospholipase. (A) *S. liquefaciens*; (B) *E. coli*(pMG200). Cells were grown in minimal AB medium containing 1% Casamino Acids and, in the case of the *E. coli* culture, 0.1 mg of AMP per ml. Symbols: ●, growth measured as OD_{450}; (○) phospholipase activity per milliliter of culture determined by use of the enzyme diffusion assay described in Materials and Methods and expressed as the fraction of maximal activity obtained in the outgrown culture.

same in logarithmically growing cells and in cells in the late growth phase (low and high OD_{450}, respectively). From these control measurements, we conclude that the cell fractionations were successful and comparable to analogous published data and that under the growth conditions used (low and high cell density), no significant cell lysis could be observed. Therefore, the distribution of phospholipase in the various cell compartments should reflect the true destination of this enzyme rather than a consequence of methodological errors.

The data concerning phospholipase produced two findings. First, no activity was measurable from the exponentially growing cells (low OD_{450}) of *S. liquefaciens* or *E. coli*(pMG200). Second, in both bacteria the enzyme activity was found almost exclusively (80%) in the growth medium. Therefore, these measurements strongly suggest that both regulation of gene expression and the excretion pathway specific for the *Serratia* phospholipase are present in the two bacterial species.

Genetic mapping of the phospholipase gene. The position of the *phl*$^+$ gene in the 3.2-kbp *Eco*RI fragment was determined by subcloning. The results of this analysis (Fig. 1) show that the gene must be located between the left *Eco*RI site and the *Fsp*I site (pMG201 and pMG201Δ*Sal*I). The orientation of

TABLE 1. Relative distributions, of β-galactosidase, β-lactamase, and phospholipase in host cells

OD_{450}	Activity[a]								
	β-Galactosidase (OD_{420}/OD_{450} per min per ml)			β-Lactamase (OD_{486}/OD_{450} per min per ml)			Phospholipase (mm/OD_{450} per h per ml)		
	Sup	Peri	Sph	Sup	Peri	Sph	Sup	Peri	Sph
E. coli 1100(pMG200)									
0.46	0.10	0.10	0.80	0.01	0.94	0.05	0	0	0
1.80	0.09	0.04	0.87	0.02	0.97	0.01	0.79	0.08	0.13
E. coli 1100(pNU121)									
0.42	0.05	0.15	0.80	0.08	0.85	0.07			
2.00	0.01	0.03	0.96	0.03	0.94	0.03			
S. liquefaciens									
0.52							0	0	0
1.80							0.81	0.09	0.10

[a] All activities are expressed as fractions of total activity. Sup, Peri, and Sph refer to supernatant, periplasm, and spheroplast fractions, respectively, prepared as described in Materials and Methods. Cultures were inoculated at an OD_{450} density of 0.01 and grown in LB medium. Ampicillin was added to the *E. coli* cultures. Viable counts, determined as number of bacteria per ml able to grow on LB plates and LB-plus-ampicillin plates of the outgrown cultures, were 1.4×10^9 (100% ampicillin resistant) for *E. coli* 1100(pMG200) and 0.9×10^9 (98% ampicillin resistant) for *E. coli* 1100(pNU121).

the gene was determined from plasmids constructed by insertion of a *Bal*31-digested *Eco*RI fragment in the expression vector pMG300. Cells harboring plasmids pMG323 and pMG323ΔS/E were grown on LB egg yolk indicator plates overnight at 30°C, followed by incubation at 42°C for 1 h. At this temperature, the lambda p_R promoter in the vector is induced and strong transcription will enter the inserted fragment. These clones expressed increased enzyme activity (Fig. 1), which suggests that the *phl*$^+$ gene is transcribed from left to right in the map of pMG200. We conclude that the start of the gene is just upstream of the left *Pst*I site, whereas the end of the gene must be located between the *Sal*I and *Fsp*I sites. The size of the *phl*$^+$ gene should therefore be between 750 and 1,100 bp, corresponding to a polypeptide of 250 to 370 amino acids.

DNA sequencing. The entire *Eco*RI fragment of pMG200 was sequenced by the dideoxy-chain termination method of Sanger et al. (15) (see Materials and Methods). The sequence from the left-hand *Eco*RI site to base 2300 is shown in Fig. 3, in which the orientation corresponds to the orientation of the genetic map in Fig. 1. A shotgun cloning strategy in M13 combined with cloning and sequencing of a few fragments whose orientation was known was used to obtain the DNA sequence of the 3.2-kbp *Eco*RI fragment; however, only the first 2,300 bp was sequenced completely from both strands.

The DNA was very GC rich (Fig. 3), which often made interpretation of the sequencing autoradiograms difficult because of the formation of secondary structures in the running gels. Examples of such difficult stretches are the sequences from positions 600 to 650, from positions 700 to 750, from positions 1760 to 1860, and from positions 1900 to 1960. In these cases, each strain was sequenced more than once to obtain the correct base composition. The use of deazo-dGTP in the sequencing reactions reduced this problem to some extent.

The DNA sequence indicated the presence of three reading frames. The first spanned the left *Eco*RI site to base coordinate 321, the second ran from base coordinate 508 to the stop codon at 1465, and the third ran from the ATG codon at 1461 to the stop codon at 2140 (Fig. 3). It appeared that frames two and three were overlapping but in different translational frames. The predicted molecular sizes of the proteins encoded from frames two and three are 34 and 24 kilodaltons (kDa), respectively, with codon usages very similar to those of the highly expressed *E. coli* ribosomal proteins (13).

The position and orientation of the second open reading frame, corresponding to the 34-kDa polypeptide, matched the genetic mapping of the phospholipase gene (see above), and the presence of the sequence AAGGAG (resembling a ribosomal binding site) 5 bp upstream of the ATG codon at coordinate 508 supports the suggestion that this putative polypeptide is the phospholipase (phlA in Fig. 1 and 3). The overlapping downstream open reading frame (24 kDa) is designated by the genetic symbol *phlB*.

The two putative polypeptides both contained hydrophobic amino termini, which might function as signal or transmembrane peptides. Compared with other signal peptides of periplasmic and membrane-associated proteins, a striking difference was the lack of charged amino acids such as lysine and arginine in the signal of the 34-kDa protein, i.e., the phospholipase. The DNA and amino acid sequences were also compared with the sequence of the *E. coli*-encoded phospholipase A located in the outer membrane (3). No homology at the protein level was found.

Determination of the transcriptional start site. The search for consensus promoter sequences upstream of the *phlA* structural gene gave no clear indication of a possible transcription initiation site. We therefore cloned the 200-bp *Bam*HI-*Pst*I fragment from pMG329 (Fig. 3), containing the upstream region and the first 10 codons of *phlA*, into the translational fusion vector pNM481 (Fig. 1). This construction, pMG230, resulted in an in-frame fusion between *phlA* and *lacZ*, indicating the presence of translational start signals and a functional promoter within this fragment. The multilinker in pNM481 allowed the isolation of an *Eco*RI-*Hin*dIII fragment containing the 200-bp *Serratia* fragment. This fragment was used as a probe in an S1 nuclease analysis of the transcriptional start site. Total RNA was isolated from cultures of MT102 containing pMG200 or (as a control) pNU121 at a cell density of approximately 1.5 OD_{450} units as described in Materials and Methods. We found from the S1 treatment that transcription was initiated from base coordinates 460 and 461 (Fig. 3).

Identification of the *phlA* and *phlB* gene products. The nucleotide sequence (Fig. 3) indicated the presence of two genes whose products would be polypeptides of 34 kDa (319 amino acids) and 24 kDa (227 amino acids), respectively, and all of the data described so far are consistent with the suggestion that the 34-kDa product is the phospholipase enzyme. To demonstrate directly that the two proteins were indeed expressed from the cloned fragment, an in vitro protein-synthesizing system was used. During this analysis, we found that the *phl* promoter was not active in our S-30 extract prepared from exponentially growing *E. coli* cells; therefore, the results presented in Fig. 4 through 7 were obtained from templates in which the lambda p_R promoter transcribed into the 319-amino-acid reading frame (using the vector pMG300 described above).

Several of the *Bal*31-generated fragments inserted into pMG300 (pMG311, pMG322, and pMG323; see Fig. 3 for genetic maps) directed the in vitro synthesis of a 34-kDa polypeptide (Fig. 4). Template pMG305 directed the synthesis of both a 34-kDa and a 24-kDa polypeptide (Fig. 5). Identical results were obtained with templates pMG330 and pMG329 (not shown). Both plasmids carried approximately 2 kbp of the *Eco*RI fragment; i.e., they carried just sufficient DNA to cover both the 34-kDa and 24-kDa reading frames. With pMG323ΔS/E as a template, only the 34-kDa product was synthesized; with pMG305Δ*Sal*I, only the 24-kDa protein was expressed (Fig. 6). It should be noted that only the orientation of the *Eco*RI-*phl*$^+$ fragment in pMG305 (in contrast to that in pMG306) resulted in synthesis of the two proteins (Fig. 5). Moreover, phospholipase activity was also expressed in the in vitro system as determined by the egg yolk assay. After blotting the polypeptides from the SDS-polyacrylamide gel onto an overlayered agarose gel containing dissolved egg yolk, it was observed that the 34-kDa band comigrated with phospholipase activity (Fig. 7).

From two of the added templates (pMG313 and pMG317), larger polypeptides were expressed in vitro (38 and 36 kDa, respectively); from pMG314, a 28-kDa polypeptide band was seen (Fig. 4). According to the nucleotide sequence (Fig. 3), pMG313 and pMG317 carry in-frame fusions between the first 28 amino acids of the lambda Cro protein (present in pMG300) and the PhlA protein. The molecular weights of 38 and 36 kDa fit the expected sizes of such fusion proteins. The *phl* fragment present in pMG314 represents a deletion of 60 amino acids of the *phlA* coding sequence which is not in frame with the *cro* sequence. Although the size of 28 kDa would be expected from such a deletion, we do not know at present where the exact translational starting point is.

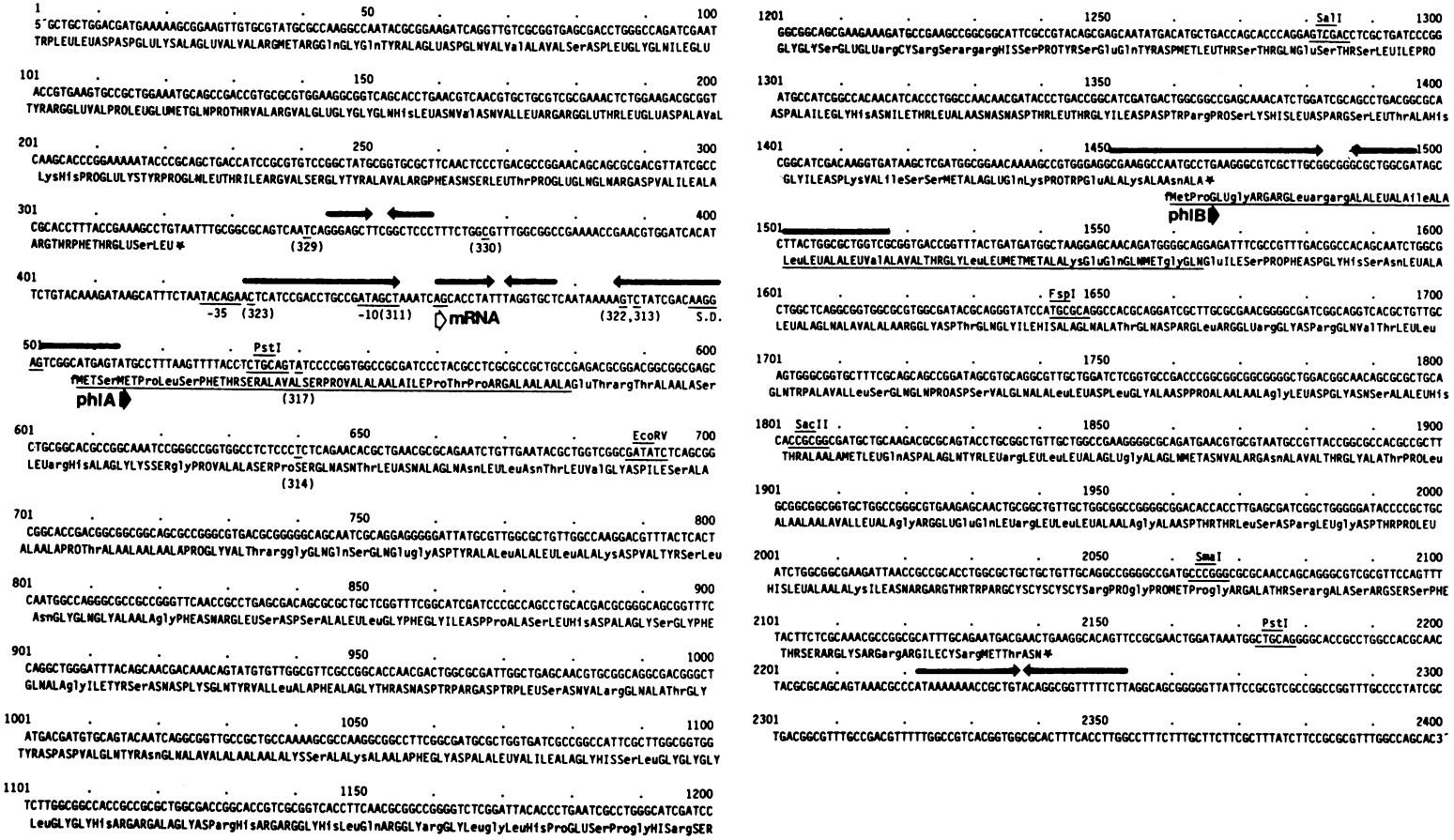

FIG. 3. DNA sequence of part of the 3.2-kbp *S. liquefaciens Eco*RI fragment encoding phospholipase activity. Arrows indicate inverted repeats; asterisks indicate translational stop codons; S.D., Shine-Dalgarno homology. Putative RNA polymerase −35 and −10 sequences and putative signal sequences are underlined; mRNA indicates site of transcriptional initiation; codons in capital letters indicate codon usage similar to that in the well-expressed *E. coli* ribosomal genes. Restriction sites of important restriction enzymes are shown, as are the extents of *Bal*31 digestion, from left to right (single-letter underline), of the plasmids pMG329, pMG330, pMG323, pMG311, pMG313, pMG317, and pMG314.

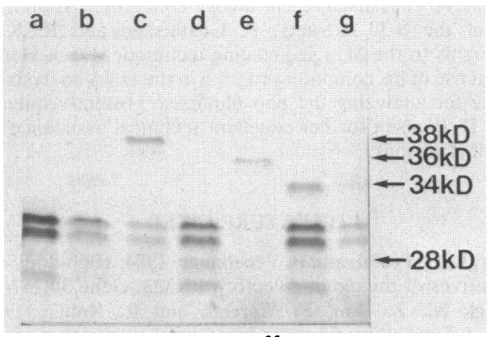

FIG. 4. Autoradiogram showing ^{35}S-labeled proteins synthesized in the Zubay system (21) and resolved on a 15% polyacrylamide gel. Templates used were (a) pMG300, (b) pMG311, (c) pMG313, (d) pMG314, (e) pMG317, (f) pMG322, and (g) pMG323. Arrows indicate molecular sizes of *cro-phlA* fusion products (c and e), truncated product (d), and normal *phlA* product (b, f, and g). The positions of enzyme activity in the gels were detected by using the phospholipase activity-blotting technique described in Materials and Methods.

Enzymatically active protein was expressed even from these three templates both in vivo and in vitro, which shows that significant alterations in the amino-terminal end of the phospholipase are without serious effects on activity. This finding enabled us in all cases (Fig. 4 through 7) to identify hybrid and wild-type phospholipase in the gels.

DISCUSSION

The exoenzyme phospholipase A1 is one among several extracellular enzymes excreted to the outside environment by different *Serratia* spp. We are interested in the excretion processes among gram-negative bacteria, in particular those belonging to the family *Enterobacteriaceae*. An important aspect of the analysis of the excretion capacity of *Serratia* spp. is the comparison with *E. coli*, which normally does not seem to be able to totally excrete proteins to the growth medium. Therefore, the cloning and characterization in *E. coli* of the phospholipase A1 gene from *S. liquefaciens* presented here is an important step toward our final goal.

It is often found that cloning in *E. coli* of exoprotein genes from other bacteria does not lead to a complete export of the protein but in many cases to a translocation to the periplasmic space. It is therefore surprising to us that the *Serratia* phospholipase apparently maintained its extracellular destination in *E. coli*. Because of the enzymatic activity, of this protein, however, it could be argued that cell lysis was a likely explanation for the excretion; we therefore performed careful measurements to investigate this possibility.

The distribution analysis (Table 1) of cytoplasmic and periplasmic marker enzymes shows that the release of phospholipase from *E. coli* cells to the medium is not related to cellular lysis. We are therefore convinced that this protein is subject to full excretion even in *E. coli*.

Most described periplasmic and outer-membrane-bound proteins of *E. coli*, and secreted proteins of bacterial and eucaryotic origin in general, have been shown to possess an amino-terminal signal sequence which, upon membrane transfer, is cleaved by specific signal peptidases, resulting in the liberation of mature protein on the outer side of the membrane. The consensus structure of such signal sequences has been established by von Heinje (19) among others. Inspection of the DNA nucleotide sequence shows that both the 34-kDa protein (the phospholipase) and the 24-kDa protein exhibit such signal peptides. One striking feature of the phospholipase is the lack of charged amino acids such as lysine or arginine at the amino terminus. However, the presence of a 17-amino-acid amino-terminal stretch with an overall hydrophobicity followed by a 7-

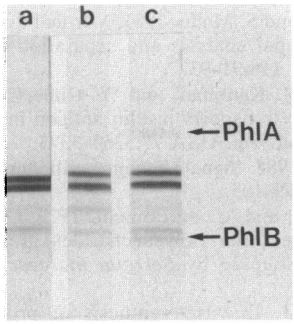

FIG. 5. Autoradiogram showing the two gene products synthesized from the 3.2-kbp *Eco*RI fragment. Conditions were as for Fig. 4. Templates used were (a) pMG300, (b) pMG306, and (c) pMG305. Arrows indicate positions of the gene products of *phlA* and *phlB*.

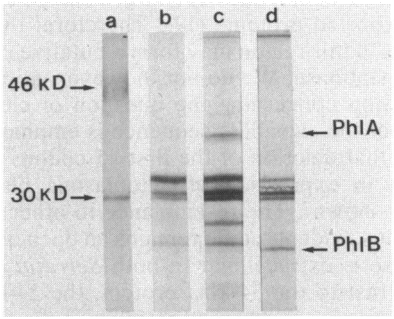

FIG. 6. Autoradiogram showing proteins synthesized from templates deleted for different restriction fragments. Conditions were as for Fig. 4. Lanes: a, molecular weight standard; b, pMG300; c, pMG323/ΔS/E; d, pMG305Δ*Sal*I.

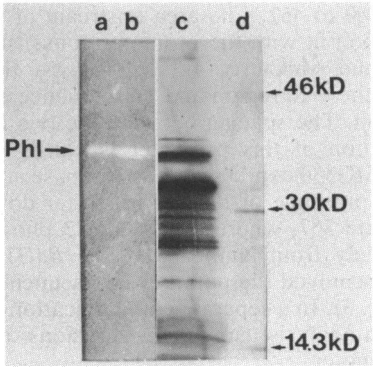

FIG. 7. Detection of phospholipase enzymatic activity in a polyacrylamide gel and comparison of migration of enzyme activity and labeled protein band. Arrows indicate the positions of white dense precipitation in the overlayered egg yolk carrying agarose (see Materials and Methods). Lanes: a, supernatant from an *E. coli* culture harboring plasmid pMG323; b, production of enzyme activity in the Zubay system (21) from pMG323; c, autoradiogram of lane b; d, molecular weight standard.

amino-acid region composed of charged and polar residues is typical of exported proteins (19). Therefore, the three alanine residues in this region may form a putative cleavage site for a signal peptidase. We do not, however, at present have any information concerning the question of cleavage. The significance of the signallike sequence is emphasized by the observation that deletion of the first 45 codons of the *phlA* gene results in expression of cytoplasmic phospholipase activity (not shown). The resemblance to other transported proteins is thus evident, and it remains an open question why phospholipase is extracellular in both *Serratia* spp. and *E. coli*. In contrast to the 34-kDa protein, the 24-kDa protein (PhlB) contains several charged residues in the amino terminus, a 19-residue highly hydrophobic stretch followed by a 7-amino-acid region of mixed hydrophobicity. No alanine residues that could form a cleavage site are present. The protein contains, in addition, an internal hydrophobic region present twice: Leu-Arg-Leu-Leu-Leu-Ala-Glu-Gly-Ala, starting at base coordinate 1831, and Leu-Arg-Leu-Leu-Leu-Ala-Ala-Gly-Ala, starting at base coordinate 1934. The function of the protein is now under investigation. In a forthcoming paper (in preparation), we will describe how this protein regulates expression of phospholipase from mRNAs initiated upstream of the *phlA* promoter at a posttranscriptional level. We have observed that deletion of the gene causes the appearance of Phl^- colonies even under antibiotic selection pressure. The gene product might be essential in fine tuning expression of the *phlA* gene under conditions of cellular growth. We have found that expression of phospholipase during exponential growth is extremely toxic to the host cells.

A common feature of many bacterial exoenzymes, including those from *Serratia* spp., is growth-phase-dependent expression. Little or no protein is synthesized in exponentially growing cells, but as the cells enter the stationary phase the genes encoding these proteins are turned on. Recently, the *E. coli*-encoded extracellular toxin microcin B17 was shown to behave in this manner (2). The phospholipase gene belongs to this class, and apparently the characteristic late expression is maintained on the cloned DNA fragment introduced in *E. coli*. This finding strongly indicates that the natural *phlA* promoter is present, active, and regulated in *E. coli* as it is in *S. liquefaciens*. S1 nuclease analysis of the transcriptional start site shows that transcription is in fact initiated upstream of the *phlA* gene at base coordinates 460 to 461. The area upstream of this position exhibits no good fit with the promoter consensus described by Hawley and McClure (4). We suggest the sequence ATAGCT as the −10 region and the sequence TACAGA as the −35 region. The spacing between the two regions is 17 bp. The position of this promoter is consistent with the finding that pMG330 expresses phospholipase at 30°C, which indicates the presence of the *phl* promoter downstream of base coordinate 357, whereas in pMG323 phospholipase is expressed solely from lambda p_R. The *Bal*31 deletion in pMG323 has removed the putative −35 sequence of the *phl* promoter (Fig. 3). In a separate communication (submitted), we present an analysis using of *lac*Z fusions of expression from this promoter.

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