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Nucleotide Sequences and Expression in *Escherichia coli* of the In-Phase Overlapping *Pseudomonas aeruginosa plcR* Genes

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The translation products of chromosomal DNAs of *Pseudomonas aeruginosa* encoding phospholipase C (heat-labile hemolysin) have been examined in T7 promoter plasmid vectors and expressed in *Escherichia coli* cells. A plasmid carrying a 4.7-kilobase (kb) DNA fragment was found to encode the 80-kilodalton (kDa) phospholipase C as well as two more proteins with an apparent molecular mass of 26 and 19 kDa. Expression directed by this DNA fragment with various deletions suggested that the coding region for the two smaller proteins was contained in a 1-kb DNA region. Moreover, the size of both proteins was reduced by the same amount by an internal *BglII-BglII* DNA deletion, suggesting that they were translated from overlapping genes. Similar results were obtained with another independently cloned 6.1-kb *Pseudomonas* DNA, which in addition coded for a 31-kDa protein of opposite orientation. The nucleotide sequence of the 1-kb region above revealed an open reading frame with a signal sequence typical of secretory proteins and a potential in-phase internal translation initiation site. Pulse-chase and localization studies in *E. coli* showed that the 26-kDa protein was a precursor of a secreted periplasmic 23-kDa protein (PlcR1) while the 19-kDa protein (PlcR2) was mostly cytoplasmic. These results indicate the expression of *Pseudomonas* in-phase overlapping genes in *E. coli*.

Coding for more than one polypeptide from a single nucleotide sequence was first found in the small DNA bacteriophages (1). Such overlapping genes have since become a relatively common arrangement in bacteriophages, viruses, and mitochondria, as well as in bacteria, which is believed to have important regulatory implications at both the level of expression and the level of protein-protein interaction (reviewed in references 12 and 20). In most cases, overlapping genes are out of phase, having a different translation reading frame (20). There are several in-phase overlapping genes in phages, due to either the readthrough of a termination codon (32) or the presence of an internal in-phase translation initiation site (8, 26). In bacteria, in-phase overlapping genes with additional initiation sites have been found in chemotactic *cheA* loci (27), in *hlyD* genes (15), in *dnaZ* and *dnaX* genes (9), and in translational *ifb* loci of *Escherichia coli* (23), as well as in genes for aspartokinase II of *Bacillus subtilis* (3).

Pseudomonas aeruginosa, unlike another gram-negative bacterium, *E. coli*, excretes many proteins across two membranes into the growth media. One of the excreted proteins, hemolysin, possesses phospholipase C enzyme activity, and its expression is controlled by phosphate regulation (2, 28). As part of our studies on the mechanism of protein excretion across the inner and outer membranes and on the control of secreted proteins by phosphate, we have cloned a 4.7-kilobase (kb) chromosomal DNA from *P. aeruginosa* strain PA103 (7, 14) and a related larger 6.1-kb DNA from strain PAO1 (24, 30) that express phospholipase C enzyme activity in *E. coli*. The *plcS* gene and a downstream gene, designated *plcR*, have been found to constitute an operon (24). In this work, we analyze the translational expression of the *plcR* gene in *E. coli*. Our results suggest that the *plcR* DNA fragment contains two in-phase overlapping genes, which code for two proteins in *E. coli*, one mainly in the cytoplasm and the other secreted across the cytoplasmic membrane.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* K-12 strain JAKE (F⁻ *thi hsdR hsdM endB metC ΔglnP*), used for translational expression of plasmids (33), was grown on minimal medium A (5), and strain MS371 (*gal thi* T1ʳ *endA sbcB15 hsdR4*), used for transformation and for preparing plasmids (14), was grown on L broth or agar with appropriate antibiotic. Ampicillin and kanamycin were used at 40 μg/ml. Cell growth was at 37°C except when plasmid pGP1-2 was carried, when growth was at 30°C.

Isolation and analysis of DNA. Plasmid DNA was prepared as described previously (6). Treatment of DNA with restriction enzymes and ligation were carried out under conditions specified by the supplier (New England BioLabs), and DNA fragments were analyzed by electrophoresis in 0.7% agarose gels (16).

Construction of plasmids. Various DNA fragments of pSL2 (14) or pDR540-*plc* (24) were cloned into appropriate restriction sites of pT7-1, pT7-2, pT7-5, or pT7-6 (29). The plasmids and relevant DNA fragments used are shown in Table 1. The identity and orientation of the DNA inserts in the recombinant plasmids were confirmed by restriction mapping.

Expression of cloned genes in pT7 plasmid vectors. The translation products of the cloned genes under the conditional control of phage T7 RNA polymerase/promoter were analyzed essentially as described before (29, 33). Strain JAKE cells containing both pGP1-2 and a pT7 recombinant plasmid were grown overnight in L broth with 40 μg each of ampicillin and kanamycin per ml at 30°C with aeration. The cultures were diluted 10-fold in medium A supplemented with 20 μg of thiamine per ml, 0.4% glucose, 20 μg methionine per ml, and kanamycin and ampicillin (each at 40 μg/ml) and were grown at 30°C for 3 h; cells were pelleted, suspended in medium A supplemented with thiamine, glu-

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TABLE 1. Plasmids used

Plasmid	Construction or comments	Reference
pT7-1	Vector, β-lactamase gene expressed	29
pT7-2	Vector, opposite transcription orientation of pT7-1	29
pT7-5	Vector, β-lactamase gene not expressed	29
pT7-6	Vector, opposite orientation of pT7-5	29
pGP1-2	Helper, RNA polymerase expressed at 42°C	29
pSL-2	A 4.7-kb *P. aeruginosa* PA103 DNA in pBR322	14
pTS3	*Eco*RV-*Bam*HI fragment of pSL2 in *Sma*I-*Bam*HI of pT7-1	This study
pTS4	Same as pTS3 but in pT7-2	This study
pTS5	Deletion of *Pst*I-*Pst*I sites of pTS3	This study
pTS6	*Pst*I-*Bam*HI fragment of pSL2 into pT7-2	This study
pTS7	Deletion of *Sma*I-*Sma*I in pTS3	This study
pTS9	Deletion of *Sph*I-*Sph*I in pTS3	This study
pTS11	Deletion of *Bgl*II-*Bgl*II in pTS3	This study
pTS17	Same as pTS3, but in pT7-5	This study
pTS18	Same as pTS6, but in pT7-5	This study
pTS19	Deletion of *Bgl*II-*Bgl*II in pTS17	This study
pDR540-*plc*	A 6.1-kb *P. aeruginosa* PAO1 DNA in pDR540	24
pTS31	*Bam*HI-*Bam*HI of pDR540-plc in *Bam*HI site of pT7-6	This study
pTS32	Same as pTS31, but opposite transcription orientation	This study
pTS34	Deletion of *Sma*I-*Sma*I of pTS32	This study
pTS35	Deletion of *Bgl*II-*Bgl*II of pTS31	This study
pTS37	Deletion of *Bgl*II-*Bgl*II of pTS34	This study

cose, and 0.01% 18 amino acids (without cysteine and methionine), and incubated at 30°C for 60 min. The cultures were shifted to 42°C for 15 min (to activate T7 RNA polymerase), rifampin was added to 200 μg/ml (to minimize *E. coli* RNA polymerase activity), and the cultures were incubated at 42°C for an additional 15 min. The cultures were then shifted to 30°C for 20 min, and [^{35}S]methionine was added to 30 μCi/ml for 5 min. The cells were harvested, the pellets were suspended in sodium dodecyl sulfate lysis buffer, and the lysates were subjected to electrophoresis in 12% acrylamide-sodium dodecyl sulfate gels (17). The dried gels were exposed directly to Kodak XAR-5 film.

Pulse-chase of expressed products. The cultures were incubated as above, except that [^{35}S]methionine was added immediately after the cultures were shifted down to 30°C for 5 min and chased with 100 μg of methionine per ml for 15 min.

Cell fractionation. The labeled cells were harvested, and the periplasmic fraction was prepared either by cold shock (19) or with EDTA-lysozyme (11, 21). The treated cells were broken by sonication, and after 1-h centrifugation at 40,000 rpm in a Beckman Ti50 rotor, the supernatant was taken as a cytosol fraction and the pellet was suspended and taken as a total membrane fraction (21).

Quantitation of radiograms. The radiograms were scanned with an LKB Zeinek Soft-Laser Scanning Densitometer and quantitated as described previously (10).

DNA sequencing. Sequences shown of DNA isolated from plasmids pSL2 (from *P. aeruginosa* PA103) and pDR540-*plc* (from *P. aeruginosa* PAO1) were determined for both strands by the method of Sanger et al. (25) and, for certain regions, the method of Maxam and Gilbert (18), as described before (24). Sequences were analyzed with a computer provided by the BIONET National Computer Resource for Molecular Biology.

Chemicals and reagents. Translational-grade [^{35}S]methionine (>1 Ci/μmol) was obtained from New England Nuclear Corp. All restriction enzymes and related materials were from New England BioLabs. Other chemicals were of reagent grade obtained from commercial sources.

RESULTS

Expression of *Pseudomonas plc* DNA in *E. coli*. Construction of *Pseudomonas plc* DNA under control of phage T7 RNA polymerase promoters (29) allowed the detection of only specific translation products of the DNA cloned in both orientations, exclusively labeled with [^{35}S]methionine after activation of T7 RNA polymerase synthesis at 42°C and treatment with rifampin to inhibit host RNA polymerase. Expression of plasmid pTS3 containing a 4.7-kb *Eco*RV-*Bam*HI fragment from plasmid pSL2 in a pT7-1 vector revealed the synthesis of an 80-kilodalton (kDa) protein, which was previously shown to be the product of the phospholipase C structural gene *plcS* (7, 14, 24), and two other proteins with molecular masses of 26 and 19 kDa, plus three products of the pT7-1 vector *bla* gene including precursor, mature form, and degraded product of β-lactamase (Fig. 1A; two of the products were faint in this figure, but see pTS9). No translation product of the same *Bam*HI-*Eco*RV (pTS4) was evident from the opposite transcriptional orientation, i.e., in the pT7-2 vector (Fig. 1A). Expression of the same DNA fragments (i.e., pTS17 and pTS10) in pT7-5 and pT7-6 vectors, which did not express *bla* gene products, confirmed this conclusion (Fig. 1B; data not shown).

The cloned 6.1-kb *Bam*HI-*Bam*HI *P. aeruginosa* PAO1 DNA was similarly constructed and expressed. In one orientation, the translation products of 80-, 26-, and 19-kDa proteins were obtained (pTS31, Fig. 1B), while in the opposite orientation it also coded for a 31-kDa protein (pTS32, Fig. 1B), in contrast to the smaller 4.7-kb DNA insert from *P. aeruginosa* PA103 (pTS18, Fig. 1B). This open reading frame protein evidently was translationally initiated from the extra 0.7-kb DNA present in pTS32 distal to the *plcS* gene, since deletion of the extra DNA proximal to *plcS* (i.e., in pTS34) had no effect on the expression of this protein (Fig. 1B and 2).

Two proteins encoded by overlapping genes. To correlate the *Pseudomonas* translation products and gene location, the translation products of cells carrying plasmids with various deletions were analyzed (Fig. 1 and 2). The structural gene of the 80-kDa phospholipase C is located just past the *Pst*I site, as shown previously (7, 14, 24), while the deletion of *Pst*I-*Bam*HI (i.e., pTS5) defined the location of 26- and 19-kDa proteins. Since the deletion of the *Sph*I-*Sph*I DNA fragment (pTS9, Fig. 1A) did not alter translation products, both 26- and 19-kDa proteins appeared to be coded by the same 1-kb *Pst*I-*Sph*I fragment. Moreover, the internal deletion of a 0.2-kb *Bgl*II-*Bgl*II fragment resulted in the reduction of both proteins by about 10,000 daltons (pTS11, Fig. 1A). Expression of pTS19 and pTS35 with the same deletion, in which *bla* gene products were not expressed, gave identical reduction in both proteins, ruling out the unlikely possibility that they might be related to *bla* gene degradation products. The results suggest that these two proteins are encoded and expressed with approximately equal efficiency by overlapping genes present in this 1-kb

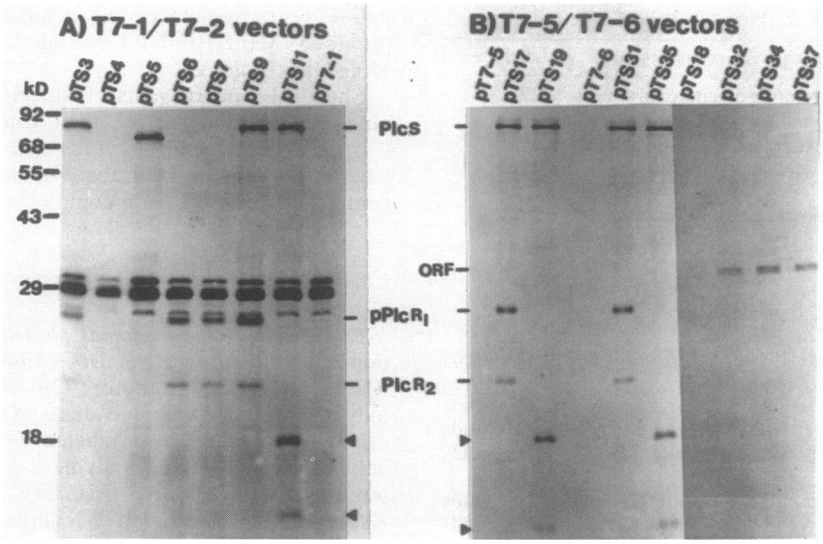

FIG. 1. Translational products of *Pseudomonas* genes in *E. coli*. Cells containing plasmid with various constructions in T7 vectors were grown and labeled with [^{35}S]methionine as described in the text. The translational products were analyzed in sodium dodecyl sulfate-gel electrophoresis and radiogramed. The arrows indicate the translational products of an internal *Bgl*II-*Bgl*II deletion. The molecular weight standards used were as follows: phosphorylase *b*, 92,000; bovine serum albumin, 68,000; heavy-chain human immunoglobulin G, 55,000; ovalbumin, 43,000; carbonyic anhydrase, 29,000; β-lactoglobulin A, 18,000.

region. The two genes are designated *plcR1* and *plcR2*; there is some evidence that they are involved in the regulation of synthesis of phospholipase C (see Discussion).

In-phase overlapping genes revealed by nucleotide sequences. The potential for overlapping genes between the *Pst*I-*Sph*I fragment (Fig. 2) was verified by nucleotide sequencing. Although not all possible reading frames are shown in Fig. 3, there is only one possible reading frame which can encode the PlcR proteins. One of the other frames contains multiple stop codons, whereas the third possible frame lacks an initiation codon (ATG or GTG). There is one open reading frame with two potential translation initiation sites, each preceded by a ribosome-binding site (Fig. 3): a strong Shine-Dalgarno sequence near the translation termination of the preceding *plcS* gene followed by an ATG codon, and a weaker Shine-Dalgarno sequence 168 nucleotide bases downstream followed by another ATG codon. Moreover, the DNA encodes two proteins in the same translation reading frame, and two *Bgl*II sites are within the structural gene and separated by 186 base pairs, correspond-

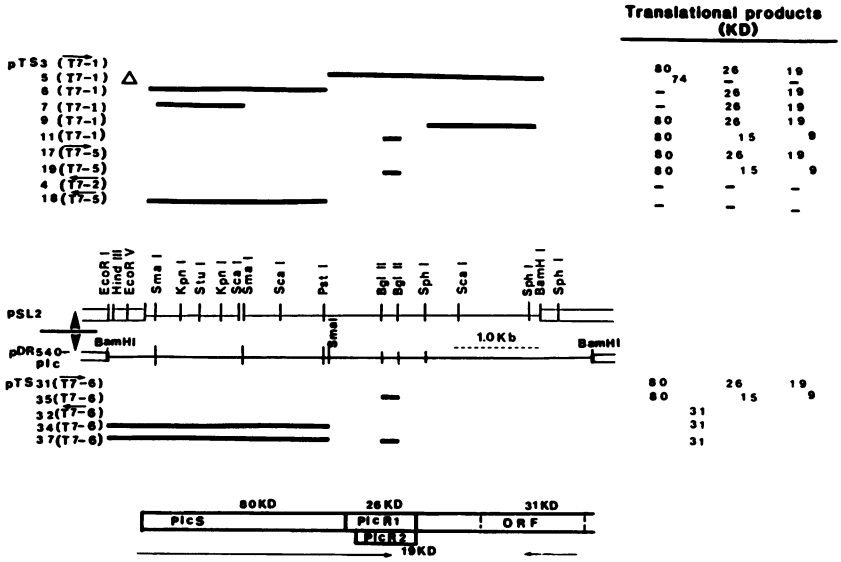

FIG. 2. Diagrammatic presentation of DNA deletion and translational products. The translational products of various plasmid construction with deletions were obtained from Fig. 1. The upper half represents the correlation of 4.7-kb *Pseudomonas* DNA in pSL2, while the lower half is that of 6.1-kb DNA in pDR540-*plc*. The arrows above T7 vectors indicate the direction of transcription/translation, and the heavy bars represent the fragment of deletion (△). The bottom part summarizes the gene organization in relation to their translational products. KD, Kilodaltons.

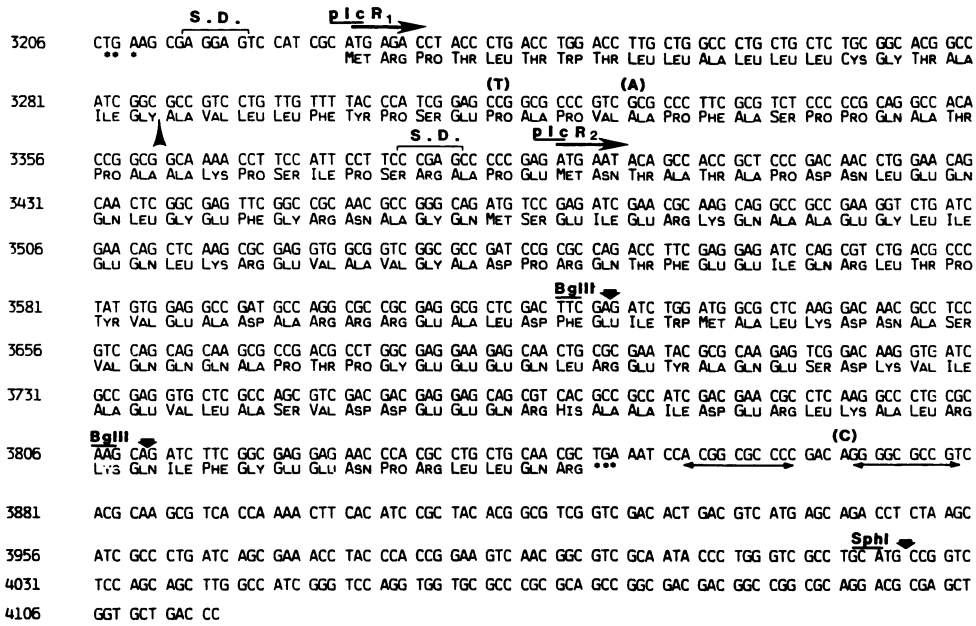

FIG. 3. Nucleotide-sequence and deduced amino acid sequence of *Pseudomonas plcR* gene. The nucleotide sequence of the nontranscribed strand of *plcR1* and *plcR2* from strain PAO1 is shown in the direction 5′ to 3′. The potential cleavage site for a signal peptidase and important restriction sites are indicated by vertical arrows. The putative Shine-Dalgarno (S.D.) regions are bracketed, and the inverted repeats for the potential transcriptional terminator are underlined with double-headed arrows. The horizontal single-headed arrows indicate the translational initiation sites of *plcR1* and *plcR2*, and the asterisks indicate the stop codons for the *plcS*, *plcR1*, and *plcR2* genes. The letters in parentheses indicate where the sequence of this region in strain PA103 varies from that of PAO1. The cytosine (C) at position 3331 was previously omitted from the partial sequence of *plcR* reported by Pritchard and Vasil (24). The numbering convention is relative to the upstream *Bam*HI site (position 1) from Pritchard and Vasil (24).

ing to 62 amino acids. Following the translation termination codon, a strong potential transcription termination site was evident ($\Delta G^\circ = -30.9$ kcal/mol), without a stretch of thymidine-rich nucleotides. The placement of the transcription terminator at this position supports earlier data from Northern analysis relating to the size of the transcript of the *plc* operon, i.e., about 2,800 base pairs (24).

There are only minor differences in the nucleotide sequences around the two *Pseudomonas plcR* genes from strains 103 (cloned in pSL2) and PAO1 (cloned in pDR540-*plc*). One nucleotide base change results in the difference of the *Sma*I site near the unique *Pst*I site in the *plcS* gene (Fig. 2 and 3).

One cytoplasmic protein and one secreted protein encoded by the overlapping genes. The amino acid sequence deduced from DNA sequences revealed that the gene product of *plcR1* contained an N-terminus sequence typical of secreted proteins (31). This precursor form (207 amino acids; 23 kDa) has a potential cleavage site at residue 20, between Gly and Ala. The gene product of *plcR2* (151 amino acids; 17 kDa) had no such signal sequence (Fig. 3). The molecular weights deduced from the DNA sequence were smaller than those (26,000 and 19,000) detected in sodium dodecyl sulfate gels, but the gel-determined molecular weights (15,000 and 9,000) of the corresponding gene products of the DNA lacking the *Bgl*II-*Bgl*II region were much closer to the sequence-derived weights (16,000 and 10,000). One distinct feature of the PlcR1 protein was the preponderance of proline residues (ten) in the region (36 amino acids) not present in the PlcR2 protein. Both PlcR proteins contained a large number of charged amino acids. The hydropathicity plot of Kyte and Doolittle (13) showed that, other than a short stretch near the signal sequence, both PlcR1 and PlcR2 proteins are quite hydrophilic (data not shown), suggesting their existence as non-membrane-soluble proteins.

Cellular localization of translational products in *E. coli*. The translation products of the cloned *Pseudomonas* genes in *E. coli* were further examined. Pulse-chase experiments revealed the existence of a 23-kDa protein, presumably the processed product of the 26-kDa PlcR1 precursors (Fig. 4, lanes 2 and 5). Labeling cells with [^{35}S]methionine right after shift-down from 42°C also allowed the detection of the 23-kDa protein (Fig. 5, lanes 3 and 4). The conversion of the 23-kDa protein from a 26-kDa precursor was also shown by the addition of solubilized membranes containing a signal peptidase activity (Fig. 5, lane 2). Localization studies of the translational products in *E. coli* showed that, although overproduction of the gene products might contribute to the imperfect cellular fractionation, 26-kDa PlcR1 precursors were exclusively present in the membrane fraction (more specifically, in the cytoplasmic membrane fraction; data not shown), the 23-kDa PlcR1 protein was mostly in the periplasmic fraction, and the 19-kDa PlcR2 protein was mostly in the cytoplasmic fraction (Table 2). The distribution of PlcS was as expected from earlier studies (14). These results were in good agreement with information deduced from DNA sequences.

DISCUSSION

Expression of the cloned *Pseudomonas plc* operon with various deletions in *E. coli* and analysis of nucleotide sequences have indicated that two proteins were encoded by in-phase overlapping genes with two translation initiation sites and were expressed with equal efficiency. If expression of such overlapping genes could be verified in *Pseudomonas*

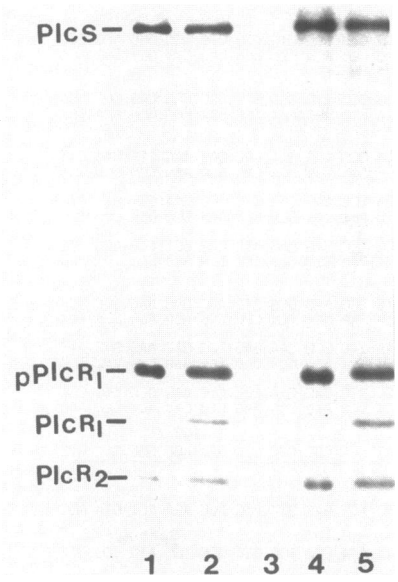

FIG. 4. Pulse and chase of translational products. Cells containing plasmids pT7-5 (lane 3), pTS17 (lanes 1 and 2), and pTS31 (lanes 4 and 5) were labeled with [^{35}S]methionine for 5 min, and portions of the cells (lanes 2 and 5) were chased with cold methionine for an additional 15 min as described in the text. The translational products were analyzed.

spp., it would join the growing list of similar gene arrangements in *E. coli* and *B. subtilis*. Pulse-chase and cellular localization studies in *E. coli* showed that, while the smaller PlcR2 protein remained soluble in the cytoplasm, the larger PlcR1 protein was mostly secreted into the periplasm across the cytoplasmic membrane after cleavage of a presumed signal peptide. Although similar cellular localization studies have not been done in the natural host, *P. aeruginosa*, the presence of a cleavable signal peptide and the hydropathicity distribution in the deduced amino acid sequence make it almost certain that the larger PlcR1 protein is either secreted into periplasm or excreted across two membranes into the growth medium in *Pseudomonas* spp. If the production of the two proteins and their compartmentation could be confirmed in *Pseudomonas* spp. this would be the first example in bacteria that in-phase overlapping genes encode two proteins that are in two different compartments, one being in cytosol and the other being secreted across the cytoplasmic membrane. In yeasts, a cytosolic invertase and a secreted invertase are encoded by overlapping genes (4).

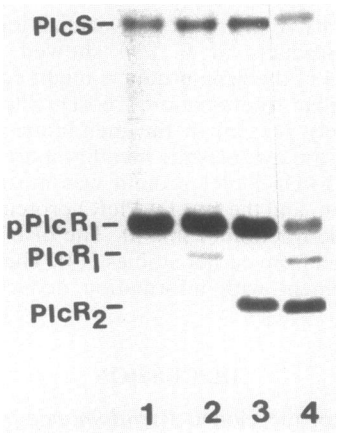

FIG. 5. Processing of the precursor of secreted PlcR1 protein. The membrane fraction from Table 2 was incubated alone (lane 1) or in the presence of 0.1% Triton X-100 and an additional 30 μg of *E. coli* membrane protein containing signal peptidase activity (lane 2) at 37°C for 10 min. Lanes 3 and 4 were product markers from Fig. 4.

TABLE 2. Localization of translational products in *E. coli*[a]

Protein (kDa)	% Protein localized in:		
	Membrane	Periplasm	Cytoplasm
PlcS (80)	71	0	28
Pre-PlcR1 (26)	99	0	1
PlcR1 (23)	0	61	39
PlcR2 (19)	0	28	72

[a] Cells containing pTS17 plasmid were labeled for 5 min after the cultures were shifted from 42 to 30°C. The periplasmic fraction was prepared by cold shock treatment twice. Similar results were obtained with the periplasmic fraction prepared by the EDTA-lysozyme method.

The functions of the PlcR proteins in *Pseudomonas* spp. are not known. Since *plcR* genes are part of an operon consisting of the *plcS* gene coding for phospholipase C, the PlcR proteins may be involved in the excretion of phospholipase C or related to phosphate regulation. Although a 186-base pair deletion mutation in the *plcR* genes has no apparent effect on expression of phospholipase C activity in *E. coli* (B. F. Shen and P. C. Tai, unpublished observations), the same mutation does affect expression of phospholipase C in *P. aeruginosa*. In a strain in which the deletion mutation has replaced the wild-type sequences in the chromosome of PAO1, phospholipase C activity is significantly increased in high- and low-P_i media, but it is still repressible by high P_i as in the wild type (R. M. Ostroff and M. L. Vasil, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, D111, p. 90). Also, there are pleiotropic effects of the deletion mutation on P_i-regulated extracellular proteins. Several extracellular proteins which are normally repressed in low P_i are derepressed in low P_i as a result of the deletion mutation (Ostroff and Vasil, unpublished observations). The preponderance of proline residues in the extra domain of the PlcR1 protein that is absent in the PlcR2 protein is also worth noting and may be related to environmental sensing, including phosphate regulation and osmoregulation.

In this work, transcription of the cloned genes was mediated by T7 promoters. The exclusive labeling of translation products of the cloned genes allowed the detection of PlcR proteins that had escaped notice earlier (7, 14, 24). Moreover, the expression in the absence of *bla* gene products revealed an open reading frame protein that would have been masked by the β-lactamase precursor (Fig. 1B). On the other hand, this work has not established the natural promoter sites for the overlapping genes. Earlier work has shown that the *plcR* gene and the preceding *plcS* gene form an operon (24). However, it is possible that the *plcR2* gene is expressed from the same RNA transcript as the *plcR1* gene or from a different internal promoter, as has been found for the yeast invertase overlapping genes (4). Further studies of a recently isolated *P. aeruginosa* mutant which has a mutation in the *plcS* gene (22) may reveal if there is an additional promoter for either of the *plcR* genes.

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