Proc. Natl. Acad. Sci. USA
Vol. 73, No. 5, pp. 1457–1461, May 1976
Biochemistry

Membrane penicillinase of *Bacillus licheniformis* 749/C: Sequence and possible repeated tetrapeptide structure of the phospholipopeptide region

(enzyme secretion/mRNA/phosphatidylserine)

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Communicated by P. Roy Vagelos, February 3, 1976

ABSTRACT **The membrane penicillinase (EC 3.5.2.6; penicillin amido-β-lactamhydrolase) of *Bacillus licheniformis* 749/C, which appears to be an intermediate in the formation of the exoenzyme, is a phospholipoprotein that carries an NH_2-terminal chain of 24 amino acids (only serine, glycine, aspartic acid, asparagine, glutamic acid, and glutamine) and a phosphatidylserine that is not present in the exoenzyme. Trypsin cleavage of the membrane enzyme produces a 26-residue phospholipopeptide whose sequence is: phosphatidylserine-Asn-Asp-Glu-Gly-Gly-Asp-Ser-Gly-Asn-Gln-Ser-Gly-Asp-Gly-Asn-Gln-Ser-Glu- Glu-Asn- Glu-Asp- Gln-Ser-Lys-COOH. This segment could be derived from a tetrapeptide [Asp(or Asn)-Glu(or Gln)-Ser-Gly] by a series of mutations (which would require reasonable base transitions and transversions), four deletions and one insertion. The putative mRNA for the peptide chain would have a high purine content (up to 80%) and a structure resembling poly(A). The phospholipopeptide is long enough to span the lipid bilayer of the membrane. Hence, the phosphatidylserine residue could be on either face of the membrane and still allow the major catalytic portion of the enzyme to be in the external aqueous phase.**

The production of penicillinase (EC 3.5.2.6; penicillin amido-β-lactamhydrolase) by *Bacillus licheniformis* 749/C has been used for investigation of the process by which a bacterial cell secretes an enzyme (1). The organism releases about half of its penicillinase into the medium; the remainder is cell bound and accessible to substrate, and much of it is associated with mesosome-like structures released during conversion of the cells to protoplasts. There is only a single structural penicillinase gene (2) from which, presumably, both enzymes are derived, and the enzyme released from the cell by trypsin differs from the exoenzyme only by the absence of the NH_2-terminal lysine (3).

Membrane penicillinase (4) differs from the exopenicillinase by the presence of a phospholipopeptide chain of 25 amino acids at its NH_2-terminus. Acid hydrolysates of the peptide contain only serine, glycine, glutamic acid, aspartic acid, and lysine. The phospholipopeptide (obtained by trypsin treatment of the membrane enzyme) has been purified and part of its structure elucidated (4): the NH_2-terminal residue is probably phosphatidylserine, and the COOH-terminus is lysine (the same residue as the NH_2-terminal lysine of the usual penicillinase). The phospholipopeptide tail appears to be responsible for the hydrophobic properties of the membrane enzyme (exopenicillinase shows no detectable affinity for the cell membrane) and could anchor the enzyme to the plasma membrane while leaving the major portion free to interact with substrate or antibody (5–8). We report here the sequence of this unique region of the membrane penicillinase.

MATERIALS AND METHODS

The phospholipopeptide preparations (molecular weight 3380–4000) were obtained from the membrane penicillinase of *B. licheniformis* 749/C by trypsinization (4). They contained fatty acids (composition similar to that of the plasma membrane lipids), 12.6–14.5% nitrogen, 0.6–0.8% phosphorous, glycerol, and amino acid residues (approximately 8.0 aspartic acid, 4.7 serine, 6.7 glutamic acid, 5.4 glycine, and 1.0 lysine, and including 4.4–9.0 amide residues on the basis of nitrogen analysis). The lipid, in the form of phosphatidylserine, is covalently bound to the protein through the phosphate and is not removed upon purification by ion exchange chromatography in the presence of Triton X-100, polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate, or exhaustive extraction with various mixtures of organic solvents of differing polarities (4, 7, 8).

For determination of the sequence from the NH_2-terminus, the subtractive and successive phenylisothiocyanate degradation procedures (9, 10), leucine aminopeptidase (EC 3.4.11.1) digestion (11), and the dansyl procedure (12, 13) were used. Identification of the phenylthiohydantoin-amino acids was made by gas chromatography (14) and by thin-layer chromatography (15, 16). The sequence from the COOH-terminus was determined by digestion with a mixture of carboxypeptidases A and B (EC 3.4.12.2 and 3.4.12.3) (11). COOH-terminal residues were also determined by the selective tritium-labeling method (17). The amide residues were detected by comparison of amino acid residues before and after glutaminase (EC 3.5.1.2) and asparaginase (EC 3.5.1.1) treatment (18) of carboxypeptidase and aminopeptidase digests, and by direct identification of the phenylthiohydantoin- and dansyl-amides through gas and thin-layer chromatography, respectively.

Digestion of Phospholipopeptide with Proteolytic Enzymes. (*a*) *Papain:* Phospholipopeptide (or peptide) was dissolved at 1 μmol/ml in 0.2 M pyridinium acetate buffer, pH 4.0, and 50 μl of 0.5% (wt/vol) papain (EC 3.4.22.2) solution in 0.01 M pyridinium acetate buffer, pH 4.0, was added per ml of peptide solution. The digestion mixture contained 0.01 M 2,3-dimercapto-1-propanol to keep the papain in the reduced form and to chelate any heavy metal ions. The mixture was incubated in a screw-capped vial at 37° for 18 hr, and the reaction was terminated by lyophilization. (*b*) *Subtilisin:* Phospholipopeptide (or peptide) at 1 μmol/ml was incubated for 15 hr at 30° in 0.1 M ammonium bicarbonate, pH 7.5, with 50 μg of subtilisin (EC 3.4.21.14) per ml. The digestion was terminated by lyophilization. (*c*) *Pepsin:* Phos-

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Table 1. Amino acid composition of fragments produced from the phospholipopeptide by papain, pepsin, and subtilisin

		Digested with								
		Papain			Pepsin		Subtilisin			
	Phospho-lipopeptide	I	II	III	I	II	I	II	III	IV
Aspartic acid	8.0	2.1	4.1	2.1	6.2	2.0	3.8	2.2	*	2.4
Serine	4.7	0.6	2.8	0.9	3.8	0.7	1.6	0.7	0.8	0.9
Glutamic acid	6.7	0.7	3.9	1.8	5.2	1.6	1.1	1.0	3.2	2.2
Glycine	5.4	*	4.9	*	4.7	*	2.9	2.3	*	*
Lysine	(1.0)	*	*	1.1	*	1.1	*	*	*	1.2
NH_2-terminus	None	None	Gly	Asn	None	Asn	None	Gln	Gln	Asn
COOH-terminus	Lys	Glu	Glu	Lys	Glu	Lys	Asn	Asn	Glu	Lys
Phosphorous	0.6–0.8%	+	—	—	+	—	+	—	—	—
Fatty acid	+	+	—	—	+	—	+	—	—	—

Values are molar ratios of amino acids recovered after hydrolysis in 6 M HCl for 20 hr at 110° (not corrected for destruction or incomplete hydrolysis). Molar ratios under 0.1 are indicated as *. Enzyme digestions and separation of peptides were carried out as detailed in the *text*. The NH_2- and COOH-termini were identified by dansylation and digestion with carboxypeptidases A and B, respectively. Phosphorous and fatty acids were detected by spotting a sample of the fragment on a Silica gel G plate and spraying with Zinzadze reagent or Rhodamine 6G.

pholipopeptide was dissolved in 0.01 M HCl at 1 μmol/ml and the pH was adjusted to 2.0 with 0.2 M HCl. A freshly prepared 1% (wt/vol) solution of pepsin (EC 3.4.23.1) in 0.01 M HCl was added to a final concentration of 0.005%. After 24 hr at 30°, the digest was lyophilized to stop the digestion.

Separation of Fragments from Enzyme Digestions. The digests were subjected to gel filtration on a 1.5 × 90 cm column of Sephadex G-50, fine grade, in 0.1 M ammonium hydroxide containing 0.1% (wt/vol) Triton X-100. Peptides were estimated by the ninhydrin reaction after alkaline hydrolysis (19). The presence of fatty acids and phosphorous was detected by spot tests on Silica gel G plates sprayed with Rhodamine 6G or Zinzadze reagent, respectively (Supelco, Inc., Bellefonte, Pa.). Peptides were also separated on Whatman 3 MM paper by peptide mapping procedures: descending paper chromatography in 1-butanol:acetic acid:water (4:1:5; vol/vol) for 15 hr was followed by high voltage electrophoresis in pyridine:acetic acid:water (3.3% acetic acid was titrated to pH 3.70 with pyridine) for about 90 min at 2000 V and 10° with the use of a Savant flat-plate apparatus (Savant Instruments, Inc., Hicksville, N.Y.). For preparative runs, 100–600 nmol of peptide were separated and the papers were sprayed lightly with 0.025% ninhydrin solution in ethanol: 2 M acetic acid (3:1, vol/vol) and developed at 80° for 5–10 min. Peptide spots appearing after spraying were cut out and eluted from the paper with 0.1 M acetic acid or 0.1 M ammonium hydroxide. Eluted peptides were lyophilized for analysis.

RESULTS

Since the peptide portion of the phospholipopeptide contains only aspartic acid (or asparagine), glutamic acid (or glutame), serine, glycine, and lysine residues, nonspecific proteases such as papain, pepsin, and subtilisin were used to obtain small overlapping peptides for sequence determination. Papain, pepsin, and subtilisin digests gave three, two, and four fragments, respectively (Table 1). In each group, one peptide had lysine as its COOH-terminus while another contained phosphorous and fatty acids (and thus the phosphatidylserine) but not a reactive NH_2-terminal residue.

Fragments Produced by Papain and Pepsin Digestion. These were chosen for the determination of amino acid sequence. The digests were subjected to gel filtration on a column of Sephadex G-50 (1.5 × 90 cm) in 0.1 M ammonium hydroxide containing 0.1% (wt/vol) Triton X-100. In each digest the fragment eluting in the void volume contained the phospholipid, and no reactive NH_2-terminal residue could be detected; the material in the included peaks did not react with the spray reagents for phosphorous and lipids. This distribution can be explained by the tendency of the phospholipid portion to complex with a micelle of detergent to produce an aggregate large enough to elute at the void volume. Thus, all fragments eluting at the void volume can probably be designated as NH_2-terminal fragments.

Hydrolysis of the phospholipopeptide by papain produced three fragments (Fig. 1A; Fig. 2). Fragment PaI eluted at the void volume and contained the phospholipid residue that has been identified as phosphatidylserine (4). Digestion of PaI with carboxypeptidases released glutamic acid most rapidly, thus identifying it as the COOH-terminus; aspartic acid occupied the penultimate position. The third amino acid was identified as asparagine by its cleavage with asparaginase after prolonged digestion of PaI with carboxypeptidases. Therefore, the sequence of PaI is phosphatidylserine-Asn-Asp-Glu-COOH (Fig. 3).

Peak PaII material (Fig. 1A) was further digested with subtilisin, and separated into three fragments (PaII-SI, PaII-SII, and PaII-SIII) by the peptide mapping procedure. Peak PaIII material contained the lysine residue known to be the COOH-terminus of the phospholipopeptide (4). This assignment was confirmed by selective tritiation of the phospholipopeptide (17) followed by papain digestion; lysine was the only amino acid labeled.

The pepsin digest of the phospholipopeptide was separated on Sephadex G-50 into two fragments (Fig. 1B). The material in the void volume peak (PeI) was treated with subtilisin (Fig. 2) and fractionated on Sephadex G-50. The material in the included peak from this fractionation gave two spots (PeI-SII and PeI-SIII) on a peptide map; the material of the excluded peak (PeI-SI) was further digested with papain, followed by separation on Sephadex G-50. Carboxypeptidase digestion of the resulting material in the void volume peak (PeI-SI-PaI) showed the peptide to have the same sequence as fragment PaI (Fig. 3). The material of the included peak (PeI-SI-PaII) gave a single ninhydrin-positive

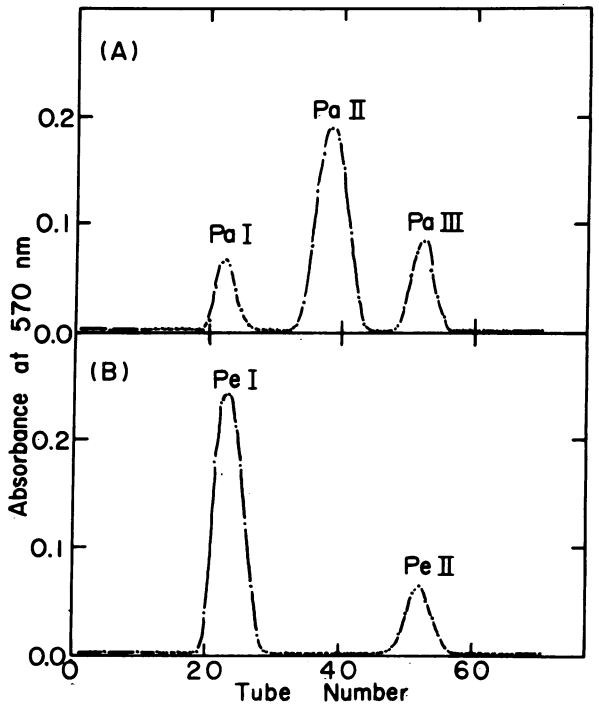

FIG. 1. Gel filtration of papaic (A) and peptic (B) hydrolysates of the phospholipopeptide on a Sephadex G-50 column (1.5 × 90 cm) equilibrated with 0.1 M ammonium hydroxide containing 0.1% Triton X-100. A 2.0-ml sample of the digest (50 mg of original phospholipopeptide) was applied and eluted at a flow rate of 20 ml/hr at 30°. Fractions of 3 ml were collected. Peptides were detected and quantitated by the ninhydrin reaction after alkaline hydrolysis (19).

spot on a peptide map. PeII, in the second peak in Fig. 1B, was identified as the COOH-terminal fragment from the phospholipopeptide, based on the presence of the lysine residue.

Total Sequence. The results for the various peptides are summarized in Fig. 3 in relation to the total derived sequence. The final amino acid composition is serine, 5; glycine, 5; aspartic acid, 4; asparagine, 4; glutamic acid, 4; glutamine, 3; and lysine 1. Thus the peptide chain contains only five residues (glycine) that are classed as hydrophobic or intermediate. All other residues are generally considered polar, including the seven with amide groups (four asparagine, three glutamine). This again indicates the importance of the phosphatidylserine residue in determining the hydrophobic properties of the membrane enzyme.

DISCUSSION

The membrane penicillinase of *B. licheniformis* 749/C consists of a hydrophilic globular protein that has a negatively charged, polar "tail" with an NH_2-terminal phosphatidylserine which is probably responsible for the hydrophobic character of the total enzyme. The peptide chain should be relatively hydrophilic and acidic since 19 of its 24 amino acid residues are polar, and these include eight dicarboxylic acids (Fig. 3); however, the phospholipid-free chain has not been available for a definitive study.

The phospholipopeptide is almost certainly long enough to extend across the lipid bilayer of the plasma membrane. Its minimum length, in the helical state, should be 3.5–4.0 nm, and its maximum, fully extended length about 9 nm (20). Since the serine and glycine residues would limit the

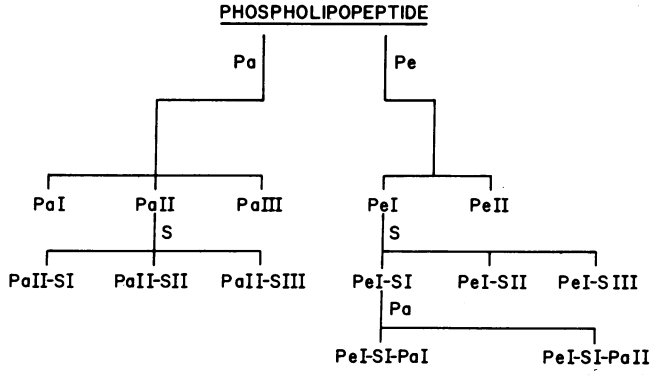

FIG. 2. Sequence of treatment of the phospholipopeptide with papain (Pa), pepsin (Pe), and subtilisin (S). See the legend of Fig. 1 and the *text* for details.

amount of helical structure, the chain should be relatively extended. Under the circumstances, the phosphatidylserine residue could be on either the outer or inner face of the membrane and still allow the major catalytic portion of the enzyme to be in the external aqueous phase, accessible to substrate and to antibodies and readily released following detachment of the phospholipopeptide by trypsin or by the endogenous releasing enzyme.

Examination of the sequence of the phospholipopeptide (Figs. 3 and 4) reveals the existence of two different segments in the peptide chain. Residues 16–26 are all polar and include four dicarboxylic acids; residues 1–15 contain the five glycines (nonpolar) and the phosphatidylserine. This differentiation should markedly affect the affinity of these segments for the membrane and hence the orientation of the total enzyme molecule.

The existence of only six types of residues in the phospholipopeptide suggested that it had arisen by reiteration and modification of an oligopeptide structure. From the arrangement presented in Fig. 4 it seems plausible that the peptide chain has been derived from an initial -Asp (Asn)-Glu (Gln)-Ser-Gly- sequence; this would account for the position of almost all of the residues. The arrangement assumes that the repeated unit occurs seven times and that there have been four deletions and one duplication.

A good test of the possible tetrapeptide origin is to examine the mutational changes that would have been required during such a process. Fig. 4 shows the mRNA codons likely to correspond to the individual residues (assuming that this segment is formed by a typical ribosomal system for protein synthesis). The conversion of Asp to Asn (or the reverse) would require a G ↔ A transition, and the Glu to Gln (or reverse) shift a G ↔ C transversion; both occur frequently (21). Thus, the assignment of these residues to the first two positions of a repeating unit is reasonable; however, there is no basis for suggesting whether the original residues were amidated. The possible codons for serine (third column, Fig. 4) are AG^U/C, UG^U/C, and UC^A/G, and those for glycine (fourth column) are GG^U/C and GG^A/G. We suggest that the original codon for serine was AG^U/C and for glycine was GG^A/G, since on this basis one can readily explain a replacement of serine by glycine at position 5 ($AG^U/C \rightarrow GG^U/C$) and of glycine by glutamic acid at position 19 ($GG^A/G \rightarrow GA^A/G$). In addition, one must postulate a duplication of the glutamic acid residue at position 19 and four deletions. We conclude that the required mutational changes permit us to propose that the phospholipopeptide was derived from an initial tetrapeptide sequence.

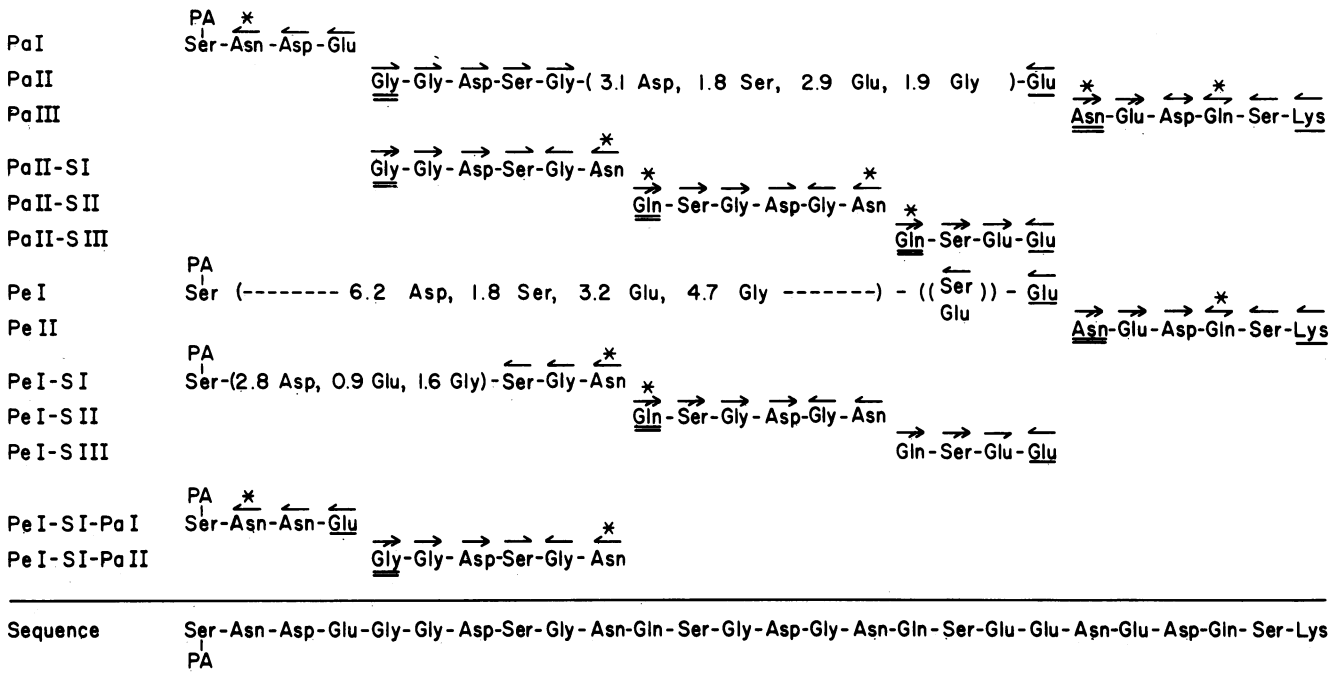

FIG. 3. Summary of sequence data for digests of phospholipopeptide with papain, pepsin, and subtilisin. (—) Subtractive Edman degradation; (→) successive Edman degradation; (⇀) leucine aminopeptidase method; (←) carboxypeptidase A and B method: (double underline) dansyl method; (underline) tritium labeling method; (*) asparaginase and glutaminase treatment after digestion with leucine aminopeptidase or carboxypeptidases A and B.

A striking feature of the proposed mRNA is its high purine content (almost 80%; Fig. 4). All codons contain at least two purines, and all but three of the pyrimidine residues occur in the third position. This 75-nucleotide fragment should have a structure very similar to that of poly(A). Since the poly(A) segment at the 3′-end of mRNAs from eukaryotic cells has been reported (22, 23) to have an affinity for membrane, the possibility arises that both the mRNA for membrane penicillinase (5′-end) and the nascent peptide chain contain structures that would be expected to favor translation of the mRNA on membrane-bound rather than on free ribosomes.

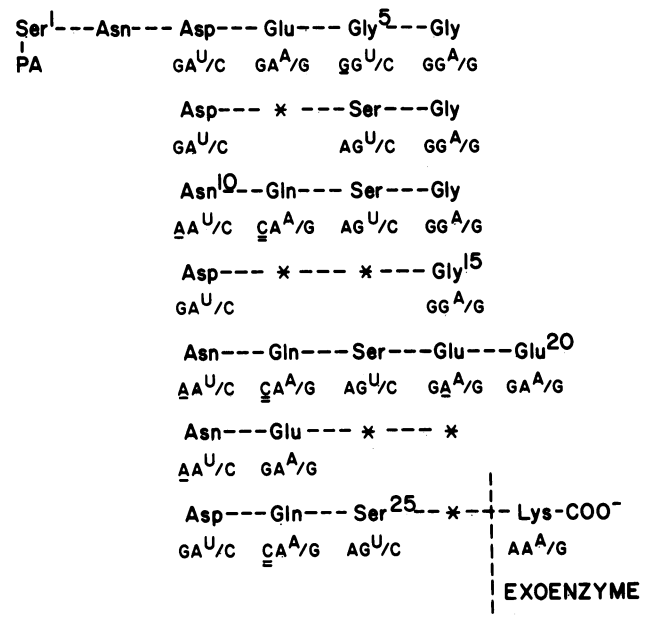

FIG. 4. The phospholipopeptide as a repeated tetrapeptide. The suggested mRNA codons corresponding to the individual residues are also shown. Symbols: (*) hypothetical deletions of amino acids; (underline) and (double underline) transitions and transversions, respectively.

Our comments concerning the mRNA codons for the phospholipopeptide are based on the assumption that this portion of membrane penicillinase is a part of the actual gene product. This concept is supported by the recent observation (24) that the only detectable penicillinase formed by an *in vitro* protein synthesis system from *B. licheniformis* 749/C is a hydrophobic enzyme containing the bound phospholipid. We infer from this that the phospholipid is attached while the nascent enzyme is bound to the ribosome and that exopenicillinase is a product of cleavage of the membrane enzyme. In this regard it should be noted that membrane penicillinase is not a unique type of protein. At least six or seven additional membrane proteins with hydrophobic tails that contain ^{3}H-labeled phosphatidylserine have been detected in uninduced *B. lichentformis* 749 cells grown in the presence of [2-^{3}H]glycerol (P. S. Aiyappa and J. O. Lampen, unpublished observations).

This work was supported by U.S. Public Health Service Grant AI-04572 from the National Institute of Allergy and Infectious Diseases.

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