INFECTION AND IMMUNITY, Dec. 1993, p. 5078–5089
0019-9567/93/125078-12$02.00/0

Vol. 61, No. 12

Cloning, Expression, and Mutagenesis of Phosphatidylinositol-Specific Phospholipase C from *Staphylococcus aureus*: a Potential Staphylococcal Virulence Factor

SHANNON DAUGHERTY AND MARTIN G. LOW*

Department of Physiology and Cellular Biophysics, College of Physicians and Surgeons of Columbia University, New York, New York 10032

Received 29 June 1993/Returned for modification 13 August 1993/Accepted 27 September 1993

Staphylococcus aureus **secretes a phosphatidylinositol (PI)-specific phospholipase C (PI-PLC) which is able to hydrolyze the membrane lipid PI and membrane protein anchors containing glycosyl-PI. The gene for PI-PLC (*plc*) was cloned from *S. aureus* into *Escherichia coli*. Oligonucleotide probes based on partial protein sequence and polyclonal antibodies raised against the purified protein were used to identify positive clones. *E. coli* transformed with a plasmid containing the *plc* gene expressed PI-PLC enzyme activity which was abolished by mutagenesis with a tetracycline resistance gene. The *plc* gene was present in all 15 *S. aureus* strains examined but not in any of 6 coagulase-negative staphylococcal species. The *plc* gene contained 984 bp and coded for a mature protein with a calculated molecular mass of 34,107 Da. Amino acid sequence comparisons indicated that the staphylococcal *plc* gene was similar (51 to 56%) to the PI-PLCs from *Bacillus cereus*, *Bacillus thuringiensis*, and *Listeria monocytogenes*. The recombinant PI-PLC expressed in *E. coli* was purified and exhibited biochemical properties identical to those of the native PI-PLC from *S. aureus*. PI-PLC production was decreased in *agr* mutant strains of *S. aureus*. However, PI-PLC production by both *agr*$^+$ and *agr* mutant strains exhibited a similar dependence on the type of medium used. These data suggested that PI-PLC production was regulated by both *agr*-dependent and *agr*-independent mechanisms.**

Staphylococcus aureus is a virulent pathogen which is responsible for a number of disease syndromes in humans and animals. It secretes a large number of proteins, several of which are known to be virulence factors while the biological role of others remains unidentified. One of the latter is phosphatidylinositol (PI)-specific phospholipase C (PI-PLC), which can affect eukaryotic cell function as a result of its ability to degrade membrane-associated inositol phospholipids. Thus, PI-PLC has been shown to mimic or interfere with cellular signalling processes (41, 48, 49, 52, 56) and to release glycosyl PI (GPI)-anchored cell surface proteins (25, 26). However, in spite of these dramatic effects on mammalian cells, the potential contribution of PI-PLC to the initiation and persistence of staphylococcal infection has received little attention.

PI-PLC activity was first identified in *S. aureus* culture supernatants over 30 years ago (8, 32). Although it was not thoroughly characterized, the early studies did suggest that the PI-PLC activity was restricted to coagulase-positive strains of staphylococci and involved in staphylococcal pathogenesis. This possibility received some support when PI-PLC activity was detected in preparations of staphylococcal delta-hemolysin (3, 62, 63). However, independent purification of the staphylococcal PI-PLC revealed it to be devoid of hemolytic activity and demonstrated that these two activities were in fact due to distinct proteins (29). Furthermore, subsequent studies with delta-hemolysin and other well-characterized staphylococcal toxins revealed that many contained traces of PI-PLC activity, but the low specific activity indicated contamination rather than molecular identity (28).

* Corresponding author.

Previous work has not demonstrated a cytotoxic role for the staphylococcal PI-PLC. A metabolic or nutritional function seems unlikely because staphylococci are not reported to contain inositol phospholipids or to require free inositol for growth. A role in the salvage of fatty acids (as a carbon source) or phosphate appears equally unlikely because *S. aureus* produces two other enzymes, a glycerol ester hydrolase (lipase) and a sphingomyelinase (beta-hemolysin), which would give the bacterium access to much greater amounts of these nutritional components in most natural environments. In *Bacillus cereus* there is evidence suggesting that sphingomyelinase but not PI-PLC is involved in phosphate retrieval (15, 57); however, similar experiments have not been performed with *S. aureus*.

Previous studies have shown that staphylococcal PI-PLC is unable to lyse erythrocytes, consistent with the apparent inaccessibility of its known substrate, PI at the cell surface (29). This is in sharp contrast to the powerful hemolytic action of two other phospholipases, *Clostridium perfringens* alpha-toxin and *S. aureus* beta-hemolysin, the substrates of which (phosphatidylcholine [PC] and sphingomyelin, respectively) are relatively abundant in the cell surface (57). However, in the last decade there have been major advances in our understanding of the diverse and important roles that inositol phospholipids play in mammalian cell function, and a reevaluation of the potential contribution of PI-PLC to staphylococcal pathogenesis is warranted.

Marques et al. have compared the production of PI-PLC by strains of *S. aureus* isolated from patients with or without toxic shock syndrome or from healthy carriers (34). Although none of the strains from healthy carriers produced PI-PLC, it was produced by 10 of the 32 pathogenic strains. There was no correlation between the production of PI-PLC

and the occurrence of toxic shock syndrome, but there was a significant association between PI-PLC production and the development of acute respiratory distress syndrome (four of four patients) or disseminated intravascular coagulation (four of five patients) as complications of staphylococcal infection (34). Although these findings are highly suggestive of a role for PI-PLC in the pathogenesis of particular staphylococcal disease syndromes, they offer no clues as to the mechanism. Furthermore, our ignorance of the genetic and environmental factors which regulate PI-PLC production in vivo and in vitro hampers a precise interpretation of such studies.

In this paper we report the cloning, characterization, and in vitro mutagenesis of the gene encoding the staphylococcal PI-PLC. We have used the cloned gene to study the expression of PI-PLC by clinical isolates of *S. aureus* as well as its regulation by the accessory gene regulator (*agr*) which controls the transcription of the several toxins and virulence factors (43). Availability of the cloned gene will facilitate future studies of the contribution of PI-PLC to pathogenesis.

MATERIALS AND METHODS

Materials. [^{3}H]inositol-labelled PI ([^{3}H]PI) and [^{3}H]choline-labelled PC ([^{3}H]PC) were purchased from New England Nuclear (Boston, Mass.) and Amersham (Arlington Heights, Ill.), respectively. [^{3}H]myristate-labelled variant surface glycoprotein ([^{3}H]VSG) was isolated from *Trypanosoma brucei* as described previously (20).

Polyclonal antibodies against purified *S. aureus* PI-PLC (500 to 600 μg per injection suspended in incomplete Freund's adjuvant) were raised in two New Zealand White rabbits by two intradermal injections separated by a 2-week interval followed by repetitive subcutaneous injection at monthly intervals. The rabbits were bled at monthly intervals 6 weeks after the first injection. After the third subcutaneous injection the amount of antigen was reduced in stages to 10 μg per injection, allowing maintenance of high antibody titer for over 18 months. Although these antibodies reacted well in immunoblots with the purified staphylococcal PI-PLC (with only weak reactivity against the *Bacillus thuringiensis* PI-PLC), they also reacted with several unidentified components in staphylococcal culture supernatants. Initially it was thought that this was due to the presence of protein A released from the bacteria. However, when $F(ab)_2$ fragments were prepared by pepsin digestion of the immunoglobulin G fraction, the problem of cross-reactivity was not substantially improved. This suggested that the initial purified antigen preparation contained trace amounts of highly immunogenic contaminants.

Bacterial strains, plasmids, phages, and culture conditions. The Newman strain of *S. aureus* (originally obtained from John Pearce, Department of Microbiology, University of Birmingham, Birmingham, United Kingdom) previously used for purification of PI-PLC (29, 30) was used to isolate the gene encoding PI-PLC. This strain has been screened occasionally for colonies which produce high levels of PI-PLC in broth culture but has been otherwise free from selective pressure. *S. aureus* RN6112 and RN4256 are interrupted at the *agr* locus by a transposon insertion (Tn*551* carrying an erythromycin resistance gene) but are otherwise identical to their parental strains, RN6390 and RN4282, respectively, and were a gift from Richard P. Novick (38, 43). Clinical isolates of *S. aureus* and coagulase-negative staphylococci were obtained from the Clinical Microbiology Service at Columbia Presbyterian Medical Center and were received on blood agar plates. Standard cultures of *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Staphylococcus saprophyticus*, *Staphylococcus sciuri*, and *Staphylococcus xylosus* were obtained from the American Type Culture Collection (Rockville, Md.). *S. aureus* strains were stored as frozen stocks in Todd-Hewitt broth (THB; BBL, Cockeysville, Md.) and 10% glycerol. Unless specified otherwise, cultures were grown in 50-ml tubes that contained 5 ml of THB. In experiments with *agr* mutants, CY medium (38, 43) was used. This medium was inoculated with 50 μl of an overnight culture and was incubated at 37°C with agitation (250 rpm) for 16 to 20 h. Plasmid pRN6680 containing the tetracycline resistance gene, *tmn*, was a gift from R. P. Novick (36). Bacteriophage λZAP, the *Escherichia coli* host strain (XL1-Blue), and helper phage (R408) were supplied by Stratagene Cloning Systems, La Jolla, Calif. Culture media and growth conditions were as detailed by the supplier. *E. coli* DH5α used for transformation of pUC19 was purchased competent from GIBCO-BRL (Gaithersburg, Md.) and was cultured according to the recommendations of the supplier.

Cloning and in vitro mutagenesis of the gene encoding PI-PLC. Genomic DNA from the Newman strain was extracted by the method of Pitcher et al. (39), cleaved with restriction enzymes *Eco*RI and *Xba*I, and separated on a 0.5% agarose gel. The DNA was transferred to Nytran (Schleicher & Schuell, Keene, N.H.) via capillary transfer and hybridized with two ^{32}P-labelled synthetic oligonucleotides (17-mers with 64- and 48-fold degeneracy) whose sequences were based on two internal peptide sequences (DDAKYY and IQWADN) derived from purified *S. aureus* PI-PLC. A 6.6-kb fragment hybridizing to the probes was purified from the agarose gel with US Bioclean (USB, Cleveland, Ohio) and ligated to *Xba*I-*Eco*RI-digested λZAP. λZAP was packaged and infected into *E. coli* according to the supplier's instructions, and plaques were lifted onto nitrocellulose filters. Replica filters were screened with the oligonucleotide probes. Positive plaques were replated and screened with polyclonal rabbit antibodies specific for purified PI-PLC. To verify that the antibody binding by positive plaques was due to expression of PI-PLC, several plaques were analyzed for their ability to produce enzyme activity. Positive phage were grown in liquid culture, and the plasmid vector pBluescript was rescued from λZAP by using a helper phage as recommended by the supplier. *E. coli* XL1-Blue transformed with the recombinant plasmids was inoculated to a 100-fold dilution of an overnight culture in Luria broth supplemented with tetracycline (12.5 μg/ml) and ampicillin (50 μg/ml) and grown for 6 h with or without 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cell pellets from 0.5 ml of culture were resuspended in 0.11 ml of 0.4% sodium deoxycholate, and PI-PLC activity was assayed as described below. One of the plasmids (pSF122) which gave the highest expression of PI-PLC activity was used to determine the nucleotide sequence of the cloned gene.

We have designated the *S. aureus* gene encoding PI-PLC production *plc*. This designation is consistent with the nomenclature recently proposed (40) for *Listeria monocytogenes* genes encoding PI-PLC (*plcA*) and PC degrading PLC (*plcB*). However, a suffix letter was not deemed necessary for the staphylococcal PI-PLC gene because the sphingomyelinase (or beta-hemolysin) has already been designated *hlb* and there are no previous reports of PC-degrading PLC being produced by *S. aureus*, an observation that has been confirmed in the present study.

Restriction fragmentation and sequence analysis of pSF122 demonstrated that the insert was only 1.4 kb,

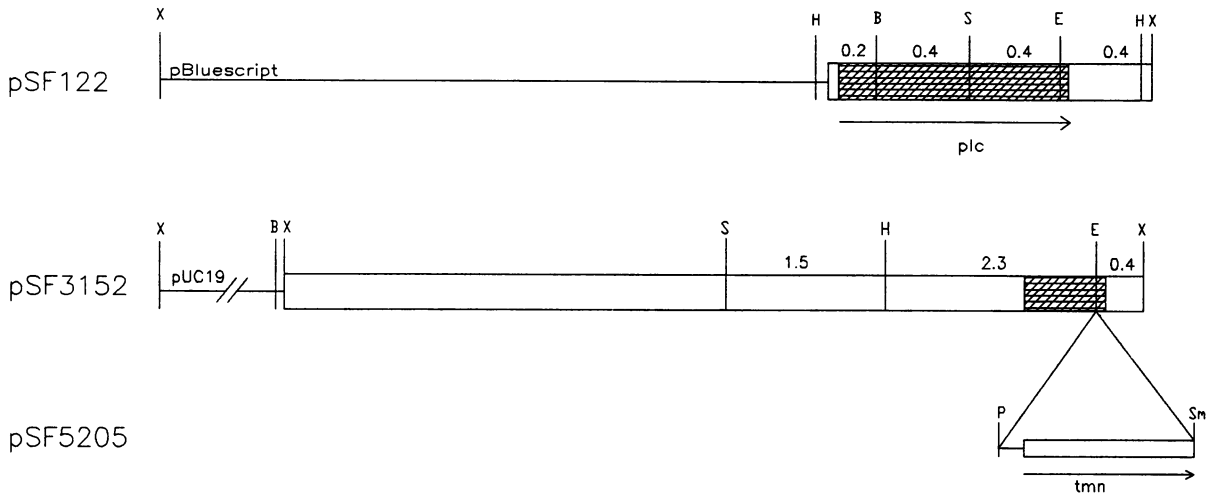


FIG. 1. Restriction map of plasmids pSF122, pSF3152, and pSF5205. Inserts of staphylococcal DNA are indicated by boxes. Crosshatched areas within boxes represent the region encoding PI-PLC which is interrupted by *tmn* in pSF5205. Approximate sizes of restriction fragments are given in kilobases. Restriction sites are indicated for *Bam*HI (B), *Eco*RV (E), *Hin*dIII (H), *Pvu*II (P), *Sma*I (Sm), *Sna*BI (S), and *Xba*I (X).

suggesting that the majority of the original 6.6-kb *Eco*RI-*Xba*I fragment was lost during rescue of the plasmid. To facilitate recombination into chromosomal DNA and preparation of *plc* mutants of *S. aureus*, a 9-kb *Xba*I fragment of *S. aureus* Newman DNA was cloned directly into *Xba*I-digested pUC19. Colony blots of transformed *E. coli* DH5α were screened by oligonucleotide hybridization and by immunoblotting. PI-PLC production was confirmed by enzyme assay. Several recombinant plasmids were analyzed by restriction mapping and found to be identical, containing 8 kb of sequence upstream and 0.4 kb downstream of *plc* (Fig. 1). The *plc* gene in one recombinant, pSF3152, was inactivated in vitro by insertion of a tetracycline resistance gene (*tmn*) which can be expressed in *E. coli*. A 3.1-kb *Sma*I-*Pvu*II fragment of pRN6680 containing *tmn* was ligated into pSF3152 linearized with *Eco*RV. The DNA was transformed into *E. coli* DH5α with selection for tetracycline (5 μg/ml) resistance. The tetracycline-resistant colonies were blotted to nitrocellulose filters and screened with an oligonucleotide probe for *plc*. Positive clones were analyzed by restriction fragmentation and Southern blot analysis and the ability to produce PI-PLC activity.

Purification of PI-PLC. PI-PLC was purified from culture supernatants of *S. aureus* Newman or *B. subtilis* BG2320 transformed with a plasmid (pMY15) containing the *plc* gene from *B. thuringiensis* as described previously (27, 30). Apart from the initial culture and the preparation of the cell lysate, the PI-PLC from *E. coli* transformed with pSF122 was purified by the same procedure. Four 2-liter Erlenmeyer flasks containing 500 ml of Luria broth (50 μg of ampicillin per ml) were inoculated with 5-ml aliquots of an overnight culture grown in the same media. The flasks were incubated in an orbital shaker (250 rpm) for 20 h at 37°C. The bacteria were harvested by centrifugation and resuspended in 400 ml of 100 mM NaCl–1 mM EDTA–50 mM Tris-HCl, pH 8 (buffer A), containing 0.1 mg of lysozyme per ml and 0.5 mM phenylmethylsulfonyl fluoride. The cell suspension was stirred at ambient temperature for 60 min, 80 ml of 1% Triton X-100 in buffer A was added, and stirring was continued for an additional 30 min. Subsequent steps were performed at 0 to 4°C. The lysed cell suspension was then centrifuged at 15,000 × *g* for 30 min. The viscous supernatant was adjusted to pH 5.3 with acetic acid, filtered through two layers of Miracloth (Calbiochem), and frozen at −20°C overnight. The thawed supernatant was refiltered with Whatman no. 1 filter paper and applied (100 ml/h) to a column (2.5 by 10 cm) of Amberlite CG-50 resin equilibrated with 150 mM NaCl–50 mM Na acetate-NaOH, pH 5.3 (buffer B). The column was washed with 300 ml of buffer B and eluted with a linear gradient consisting of 250 ml each of buffer B and 1 M trisodium citrate. The active fractions were identified by PI-PLC assay, pooled, and concentrated to 15 ml with a stirred Amicon ultrafiltration cell with a YM10 membrane. The concentrate was dialyzed overnight versus 500 ml of 50 mM Tris acetate–0.02% sodium azide, pH 7.4 (buffer C). The dialysate was adjusted to pH 7.4 with 1 M Tris-OH and centrifuged at 15,000 × *g* for 30 min. The combined supernatants from several batches were further purified by chromatography on Sephadex G-75 and Mono S (27, 30). The purified protein was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and quantified by the bicinchoninic acid protein assay (30). Approximately 1.5 mg of PI-PLC was purified from a total of 5 liters of bacterial culture.

PI-PLC assay. PI-PLC and PC-PLC activities were determined as described previously (27, 30). Fifty microliters of the resuspended cell pellets or culture supernatants, 50 μl of 100-μg/ml bovine serum albumin (BSA)–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-NaOH (pH 7.0), 200 μl of 5 mM EDTA–50 mM HEPES-NaOH (pH 7.0), and 100 μl of [^{3}H]PI (40 nmol; approximately 5,000 cpm) or [^{3}H]PC (40 nmol; approximately 60,000 cpm) suspended in 0.4% sodium deoxycholate were incubated at 37°C for 1 h (for PI-PLC) or 20 h (for PC-PLC). The reaction was terminated by adding 1.5 ml of chloroform-methanol (1:2, vol/vol), 0.5 ml of chloroform, and 0.5 ml of 1 M HCl, and the phases were separated by centrifugation for 1 min at 500 × *g*. One milliliter of the upper aqueous phase was counted by liquid scintillation counting to determine the extent of hydrolysis. Specific activities and the effect of inhibitors and pH, etc., on activity of purified PI-PLC were determined by a modification of the

incubation system described above. One hundred microliters of purified PI-PLC diluted in 10 mM HEPES-NaOH, pH 7.0, was preincubated with 100 μl of inhibitor solution or H_2O and 100 μl of 10 mM HEPES-NaOH, pH 7.0, as specified in figure legends (unless indicated otherwise, inhibitor concentrations in figures refer to the final concentration in the assay). The reaction was started by the addition of 100 μl of substrate (suspended in 0.4% sodium deoxycholate or 0.04% Triton X-100), the mixture was incubated for 10 min at 37°C, and the amount of hydrolysis was determined as described above. In some assays, [^{3}H]VSG (20 pmol; approximately 10,000 cpm) was used as a substrate and the volume of the various additions was reduced to 50 μl but otherwise the composition was the same. VSG degradation was assessed by extraction of the incubation mixture with 1 M NH_4OH-saturated butanol as described previously (27, 30). For determination of specific activities the PI-PLC was diluted in 0.1% BSA–10 mM HEPES-NaOH to minimize surface adsorption and denaturation. One unit of activity equals 1 μmol of substrate degraded min^{-1}.

Identification of products of hydrolysis. [^{3}H]inositol phosphates liberated from [^{3}H]PI were extracted and analyzed by chromatography on Dowex AG1-X8 formate minicolumns (50). The identification of the ^{3}H-labelled product as inositol 1,2-cyclic phosphate was based on (i) its elution position in 50 mM ammonium formate and (ii) its conversion to inositol monophosphate by treatment with 1 M HCl for 3 min at 80°C. As a control, parallel incubations were performed with *B. thuringiensis* PI-PLC, which is known to produce inositol 1,2-cyclic phosphate as its initial reaction product (60). The products liberated by *S. aureus* and *B. thuringiensis* PI-PLCs behaved identically in these analyses.

The ^{3}H-labelled lipid product of [^{3}H]VSG hydrolysis was analyzed by thin-layer chromatography using Silica 60A (Whatman) developed in petroleum ether-diethyl ether-acetic acid (80:20:1, by volume). The ^{3}H-labelled product was located by fluorography after spraying the plate with En^{3}Hance. It was identified as dimyristoylglycerol by comparison with the elution positions of dimyristoylglycerol, monomyristoylglycerol, and myristic acid.

DNA and RNA blotting. Genomic DNA extracted from *S. aureus* and other staphylococcal species by the method of Pitcher et al. (39) was cleaved with *Sna*BI and *Hin*dIII and separated on a 0.5% agarose gel. DNA was transferred to a nylon filter by capillary transfer and visualized by hybridization with an ECL random-prime-labelled (Amersham) *Hin*dIII fragment of pSF122 containing the entire *plc* gene. RNA was isolated from 6-h cultures by the method of Sandler and Weisblum (54). In preliminary experiments, RNA isolated from 6-h cultures of the Newman strain resulted in the strongest signal on a Northern (RNA) blot compared with RNA signals from 4- or 24-h cultures. The yield of PI-PLC activity was approximately the same at 6 and 24 h. The RNA was separated on a 1% formaldehyde gel, transferred to a nitrocellulose filter, and visualized by hybridization with the ^{32}P-labelled *Hin*dIII fragment of pSF122 labelled with the GIBCO BRL random-primer DNA-labelling system (53).

DNA sequence analysis. Nucleotide sequences were determined in both directions with the Sequenase 2.0 DNA sequencing kit (USB). The primers used were the degenerate oligonucleotides based on peptide sequence (described above), exact oligonucleotides based on determined nucleic acid sequence (synthesized by the Protein Chemistry Core Facility of Columbia University), or universal primers (T3 and T7) purchased from Stratagene.

TABLE 1. Expression of PI-PLC activity by *E. coli* transformants

Plasmid	PI-PLC activity (mU ml^{-1})[a] in expt:	
	I	II
pBluescript	<0.1	<0.1
pSF121	>146	>118
pSF122	>140	>160
pUC19	<0.1	<0.1
pSF3152	13	17
pSF5201	<0.1	<0.1
pSF5205	<0.1	<0.1

[a] Values are means from duplicate determinations. Results of two independent experiments are given. <, value is below detection limit; >, value is outside linear range of assay and is therefore an underestimate.

DNA sequences were analyzed by using the Genetics Computer Group (version 7) software package. Sequence similarity between pairs of bacterial PI-PLCs was determined by using the BESTFIT program, and multiple sequences were aligned by using PILEUP. The sequence of the staphylococcal *plc* gene was also compared against the GenBank bacterial data base (release 75.0).

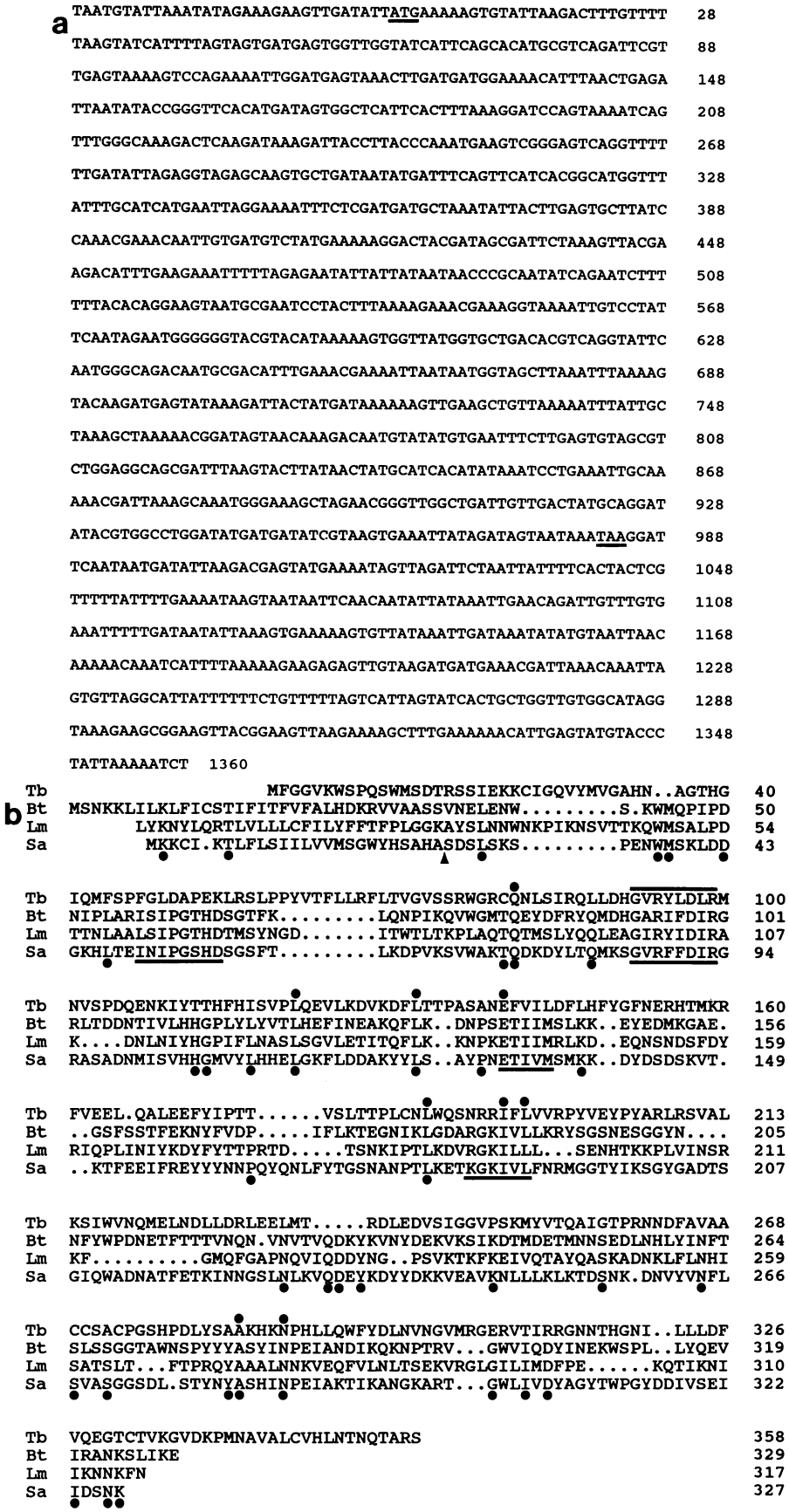
Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to GenBank and assigned the accession number L19298.

RESULTS

Cloning, subcloning, and in vitro mutagenesis of the *plc* gene. *E. coli* XL1-Blue transformed with rescued phagemid (that screened positive with the oligonucleotide probes and anti-PI-PLC antibodies) was shown to produce PI-PLC activity (Table 1). By contrast, bacteria transformed with pBluescript alone expressed no activity. Addition of IPTG to the cultures had no effect on the amount of PI-PLC produced (data not shown), indicating that the cloned gene contained all of the necessary regulatory elements. It was subsequently determined that the *plc* gene was in the opposite orientation of the *lac* operon of pBluescript. One recombinant, pSF122, containing a 1.4-kb insert (Fig. 1) was used for sequencing of the *plc* gene and for purification of PI-PLC (see below).

To facilitate recombination into chromosomal DNA and preparation of *plc* mutants of *S. aureus*, a 9-kb fragment of *S. aureus* Newman DNA, encoding PI-PLC, was cloned directly into pUC19 to generate pSF3152. The staphylococcal tetracycline resistance gene, *tmn*, was inserted into the *Eco*RV site of pSF3152 and transformed into *E. coli* DH5α (Fig. 1). Southern blotting of two tetracycline-resistant mutants (SF5201 and SF5205) showed that the *Xba*I restriction fragment which contained *plc* had increased in size from 9 to 12 kb, consistent with the insertion of *tmn*. This was confirmed by loss of the ability to express PI-PLC activity (Table 1), by Southern blotting with *tmn* as a probe, and by sequencing (data not shown).

Nucleotide and amino acid sequence of *plc*. The nucleotide sequence of the *plc* gene on pSF122 was determined and found to consist of 984 bp (Fig. 2a). This sequence predicts an open reading frame of 327 amino acid residues which contains the two 28-residue peptide sequences determined from the purified PI-PLC isolated from *S. aureus* Newman (residues 109 to 136 and 194 to 221; not indicated in figure). The *S. aureus* PI-PLC sequence was compared with sequences in the GenBank data base, but apart from the PI-PLCs from *B. thuringiensis* (17), *B. cereus* (22), and *L.*


a
TAATGTATTAAATATAGAAAGAAGTTGATATTATGAAAAAGTGTATTAAGACTTTGTTTT 28
TAAGTATCATTTTAGTAGTGATGAGTGGTTGGTATCATTCAGCACATGCGTCAGATTCGT 88
TGAGTAAAAGTCCAGAAAATTGGATGAGTAAACTTGATGATGGAAAACATTTAACTGAGA 148
TTAATATACCGGGTTCACATGATAGTGGCTCATTCACTTTAAAGGATCCAGTAAAATCAG 208
TTTGGGCAAAGACTCAAGATAAAGATTACCTTACCCAAATGAAGTCGGGAGTCAGGTTTT 268
TTGATATTAGAGGTAGAGCAAGTGCTGATAATATGATTTCAGTTCATCACGGCATGGTTT 328
ATTTGCATCATGAATTAGGAAAATTTCTCGATGATGCTAAATATTACTTGAGTGCTTATC 388
CAAACGAAACAATTGTGATGTCTATGAAAAAGGACTACGATAGCGATTCTAAAGTTACGA 448
AGACATTTGAAGAAATTTTTAGAGAATATTATTATAATAACCCGCAATATCAGAATCTTT 508
TTTACACAGGAAGTAATGCGAATCCTACTTTAAAAGAAACGAAAGGTAAAATTGTCCTAT 568
TCAATAGAATGGGGGGTACGTACATAAAAAGTGGTTATGGTGCTGACACGTCAGGTATTC 628
AATGGGCAGACAATGCGACATTTGAAACGAAAATTAATAATGGTAGCTTAAATTTAAAAG 688
TACAAGATGAGTATAAAGATTACTATGATAAAAAAGTTGAAGCTGTTAAAAATTTATTGC 748
TAAAGCTAAAAACGGATAGTAACAAAGACAATGTATATGTGAATTTCTTGAGTGTAGCGT 808
CTGGAGGCAGCGATTTAAGTACTTATAACTATGCATCACATATAAATCCTGAAATTGCAA 868
AAACGATTAAAGCAAATGGGAAAGCTAGAACGGGTTGGCTGATTGTTGACTATGCAGGAT 928
ATACGTGGCCTGGATATGATGATATCGTAAGTGAAATTATAGATAGTAATAAATAAGGAT 988
TCAATAATGATATTAAGACGAGTATGAAAATAGTTAGATTCTAATTATTTTCACTACTCG 1048
TTTTTATTTTGAAAATAAGTAATAATTCAACAATATTATAAATTGAACAGATTGTTTGTG 1108
AAATTTTTGATAATATTAAAGTGAAAAAGTGTTATAAATTGATAAATATATGTAATTAAC 1168
AAAAACAAATCATTTTAAAAAGAAGAGAGTTGTAAGATGATGAAACGATTAAACAAATTA 1228
GTGTTAGGCATTATTTTTTCTGTTTTTAGTCATTAGTATCACTGCTGGTTGTGGCATAGG 1288
TAAAGAAGCGGAAGTTACGGAAGTTAAGAAAAGCTTTGAAAAAACATTGAGTATGTACCC 1348
TATTAAAAATCT 1360
b
Tb MFGGVKWSPQSWMSDTRSSIEKKCIGQVYMVGAHN..AGTHG 40
Bt MSNKKLILKLFICSTIFITFVFALHDKRVVAASSVNELENW.....S.KWMQPIPD 50
Lm LYKNYLQRTLVLLLCFILYFFTFPLGGKAYSLNNWNKPIKNSVTTKQWMSALPD 54
Sa MKKCI.KTLFLSIILVVMSGWYHSAHASDSLSKS.....PENWMSKLDD 43
Tb IQMFSPFGLDAPEKLRSLPPYVTFLLRFLTVGVSSRWGRCQNLSIRQLLDHGVRYLDLRM 100
Bt NIPLARISIPGTHDSGTFK.....LQNPIKQVWGMTQEYDFRYQMDHGARIFDIRG 101
Lm TTNLAALSIPGTHDTMSYNGD.....ITWTLTKPLAQTQTMSLYQQLEAGIRYIDIRA 107
Sa GKHLTEINIPGSHDSGSFT.....LKDPVKSVWAKTQDKDYLTQMKSGVRFFDIRG 94
Tb NVSPDQENKIYTTHFHISVPLQEVLKDVKDFLTTPASANEFVILDFLHFYGFNERHTMKR 160
Bt RLTDDNTIVLHHGPLYLYVTLHEFINEAKQFLK..DNPSETIIMSLKK..EYEDMKGAE. 156
Lm K....DNLNIYHGPIFLNASLSGVLETITQFLK..KNPKETIIMRLKD..EQNSNDSFDY 159
Sa RASADNMISVHHGMVYLHHELGKFLDDAKYYLS..AYPNETIVMSMKK..DYDSDSKVT. 149
Tb FVEEL.QALEEFYIPTT.....VSLTTPLCNLWQSNRRIFLVVRPYVEYPYARLRSVAL 213
Bt ..GSFSSTFEKNYFVDP.....IFLKTEGNIKLGDARGKIVLLKRYSGSNESGGYN.... 205
Lm RIQPLINIYKDYFYTTPRTD.....TSNKIPTLKDVRGKILLL...SENHTKKPLVINSR 211
Sa ..KTFEEIFREYYYNNPQYQNLFYTGSNANPTLKETKGKIVLFNRMGGTYIKSGYGADTS 207
Tb KSIWVNQMELNDLLDRLEELMT.....RDLEDVSIGGVPSKMYVTQAIGTPRNNDFAVAA 268
Bt NFYWPDNETFTTTVNQN.VNVTVQDKYKVNYDEKVKSIKDTMDETMNNSEDLNHLYINFT 264
Lm KF.....GMQFGAPNQVIQDDYNG..PSVKTKFKEIVQTAYQASKADNKLFLNHI 259
Sa GIQWADNATFETKINNGSLNLKVQDEYKDYYDKKVEAVKNLLLKLKTDSNK.DNVYVNFL 266
Tb CCSACPGSHPDLYSAAKHKNPHLLQWFYDLNVNGVMRGERVTIRRGNNTHGNI..LLLDF 326
Bt SLSSGGTAWNSPYYYASYINPEIANDIKQKNPTRV...GWVIQDYINEKWSPL..LYQEV 319
Lm SATSLT...FTPRQYAAALNNKVEQFVLNLTSEKVRGLGILIMDFPE.....KQTIKNI 310
Sa SVASGGSDL.STYNYASHINPEIAKTIKANGKART...GWLIVDYAGYTWPGYDDIVSEI 322
Tb VQEGTCTVKGVDKPMNAVALCVHLNTNQTARS 358
Bt IRANKSLIKE 329
Lm IKNNKFN 317
Sa IDSNK 327

monocytogenes (4, 23, 35), no obvious similarity was found with other bacterial polypeptide sequences. The *S. aureus* polypeptide sequence exhibited 39% identity (56% similarity) with the *B. thuringiensis* sequence and 27% identity (51% similarity) with the *L. monocytogenes* sequence (Fig. 2b). This relatively low degree of identity between similar PLCs of different bacterial genera is not unprecedented. The PC-PLC of *B. cereus* exhibits only 28 and 39% identity with the *C. perfringens* and *L. monocytogenes* PC-PLCs, respectively (57, 59). A comparison of the *S. aureus* PI-PLC sequence with that of the staphylococcal beta-hemolysin, a sphingomyelin-specific PLC, (42, 57, 62), revealed only 20% identity (40% similarity).

The GPI-PLC from the parasitic protozoan *T. brucei* is related to the bacterial PI-PLCs by several criteria that are not shared by other eukaryotic PLCs which degrade inositol phospholipids: the ability to hydrolyze GPI, a similar molecular mass (41 kDa), and an absence of a divalent metal ion requirement (for references, see references 18 and 47). Comparison of the *S. aureus* and *T. brucei* sequences indicated that they were 22% identical (44% similar). When the bacterial PI-PLC sequences are compared, there are four short regions of 7 to 10 residues in which there is a 75% or greater similarity. One of these sequences, GXRXXDXR (where X represents amino acids with aromatic or relatively hydrophobic aliphatic side chains; Fig. 2b), is of particular interest since it exhibits an equal degree of conservation in the *T. brucei* GPI-PLC, suggesting that it may participate in substrate binding and/or catalysis. This sequence is not present in the staphylococcal beta-hemolysin.

Characterization of staphylococcal PI-PLC expressed in *E. coli*. **(i) Molecular mass.** As described above, *E. coli* transformed with pSF122 expressed PI-PLC activity and the yield (approximately 20 mU/ml of culture) was sufficient for purification and biochemical characterization. The molecular weights of the recombinant and the Newman PI-PLCs were indistinguishable by SDS-polyacrylamide gel electrophoresis. However, the mobility of the *S. aureus* PI-PLC is slightly greater than that obtained for *B. thuringiensis* PI-PLC (Fig. 3), which suggests that it has a slightly lower molecular mass (32 versus 33.3 kDa). A similar difference of 1 to 2 kDa was obtained in a previous study comparing the *S. aureus* and *B. thuringiensis* PI-PLCs (30). The N-terminal sequences were determined to be XXSLSKSPEN (where X equals unidentified residues) and SDSLSKS for the Newman- and pSF122-derived PI-PLCs, respectively. This indicates that in both *E. coli* and *S. aureus* a 26-residue peptide was removed from the amino terminus, producing a 301-residue peptide with a calculated molecular mass of 34,107 Da (compared to 34,511 Da for *B. thuringiensis*). The signal peptide exhibits many of the features typically found at the N termini of secreted proteins: a positively charged amino-terminal region, a relatively hydrophobic core of 9 residues, and alanine residues at the −3 and −1 sites (61).

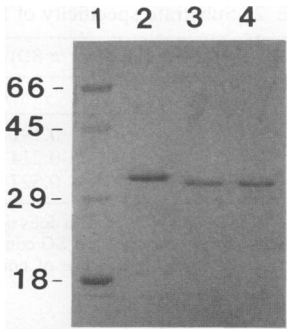


FIG. 3. SDS-PAGE of purified PI-PLC. Lane 1, molecular mass standards; lane 2, *B. thuringiensis* PI-PLC (1.8 μg); lane 3, PI-PLC (1.4 μg) purified from *S. aureus* Newman; lane 4, PI-PLC (1.6 μg) purified from *E. coli* transformed with pSF122. The gel was stained with Coomassie blue. Numbers along the side refer to the molecular masses of standards (in kilodaltons).

(ii) Specificity. Although the staphylococcal PI-PLC readily degraded [^{3}H]PI and [^{3}H]VSG (a GPI-anchored protein), it exhibited no detectable activity when [^{3}H]PC was used as substrate under the same assay conditions (Table 2). In previous studies it was shown that the staphylococcal PI-PLC had no activity towards PI 4-phosphate or PI 4,5-bisphosphate (10). The [^{3}H]-labelled products of [^{3}H]PI and [^{3}H]VSG degradation were identified as inositol 1,2-cyclic phosphate and diacylglycerol, respectively, as expected for a PLC (data not shown).

In a previous study it was noted that the specific activity of the *B. thuringiensis* PI-PLC was substantially greater than that of the PI-PLC purified from the Newman strain of *S. aureus* (30). A similar result was obtained in the present study, in which specific activity (using [^{3}H]PI as a substrate) for *S. aureus* PI-PLC derived from both the Newman strain and pSF122 was less than 10% of that observed for the *B. thuringiensis* PI-PLC derived from pMY15 (Table 2). By contrast, activity towards VSG was only about 40% of that exhibited by *B. thuringiensis* PI-PLC (Table 2). The reason for these differences in specific activity is unknown, but their occurrence in both the wild-type and recombinant PI-PLCs indicates that they are an intrinsic feature of the enzyme. Differences in specific activity for PI and VSG were noted in a previous study when PI-PLCs from *L. monocytogenes* and *B. thuringiensis* were compared (13).

(iii) Effect of pH. Previous studies by Doery et al. using crude *S. aureus* PI-PLC suggested that it had a pH optimum of 4.5 (8). It was not possible to confirm this result with the purified PI-PLC by our standard assay procedure because the detergent used for suspending the substrate (sodium deoxycholate) precipitates below pH 7. This problem was avoided by performing the assay in 0.01% Triton X-100.

FIG. 2. (a) Nucleotide sequence of *plc* gene in pSF122. Initiation and termination codons are underlined. Two potential ribosomal binding sites (GAAAGAAG and AAGAAGTT) were located at −16 to −9 and −14 to −7. An additional 184 bp were sequenced upstream (not shown) with pSF3152, but no open reading frames were found. However, a potential −10 site (TATAAT) was located at −38 to −33, and a potential −35 site (TTTACA) was located at −67 to −62. (b) Predicted amino acid sequence for *S. aureus* PI-PLC (Sa) compared with those reported for PI-PLCs from *B. thuringiensis* (Bt) (17) and *L. monocytogenes* (Lm) (35) and for GPI-PLC from the protozoan *T. brucei* (Tb) (18). The NH_2 terminus of the mature polypeptide from *S. aureus* is indicated by an arrowhead. Isolated identical residues and regions of similarity are indicated by circles and underlining, respectively. Indicators of identity and similarity appear below the sequences when they apply only to the bacterial PI-PLCs but also appear above if they apply to all four PLCs. The amino acid sequence of *B. cereus* PI-PLC (22) is 98% identical to that reported for *B. thuringiensis* and is not shown. None of the residues which differ between the two *Bacillus* sequences are located in regions of identity or similarity identified in the figure.

TABLE 2. Substrate specificity of PI-PLC

Source of purified PI-PLC	Sp act (μmol/mg/min ± SD) with:			PI/VSG[a]
	PC	PI	VSG	
pSF122	<0.01	50 ± 9.0	0.191 ± 0.046	261
Newman	—[b]	55 ± 9.5	0.214 ± 0.061	257
B. thuringiensis	<0.01	657 ± 191	0.527 ± 0.088	1,247

[a] The relatively high value of this activity ratio does not necessarily indicate that PI is the preferred substrate, because the VSG concentration (100 nM) is much lower than that of PI (100 μM) because of constraints in the assay systems.

[b] —, not determined.

Degradation of PI by either the *S. aureus* or *B. thuringiensis* PI-PLC exhibited a pH optimum of approximately 5.5 to 6.0 (Fig. 4). Activation of the *S. aureus* PI-PLC at low pH was particularly pronounced, with an approximately 20-fold increase in activity between pH 7.5 and 5.5. Similar results were obtained using VSG as a substrate, although the pH optimum was approximately 5.0. Increased PI and VSG degradation at low pH was observed with both HEPES-morpholineethanesulfonic acid (MES)-NaOH buffers (Fig. 4) and HEPES-acetate-NaOH buffers (data not shown).

(iv) Inhibition by NaCl. One property of the native *S. aureus* PI-PLC already known to distinguish it from other bacterial PI-PLCs is its extreme sensitivity to inhibition by salt solutions (30). The PI-PLCs from pSF122 and Newman exhibited identical sensitivity to NaCl inhibition (50% inhibitory concentration of 10 to 20 mM) with either PI or VSG suspended in Triton X-100 as a substrate (data not shown). By contrast, the PI-PLC from *B. thuringiensis* was relatively insensitive and required approximately 75 mM NaCl to give 50% inhibition of PI degradation and actually stimulated VSG degradation up to 150 mM NaCl. Inhibition by NaCl was also obtained with substrates suspended in sodium deoxycholate, although the effects were less pronounced (data not shown).

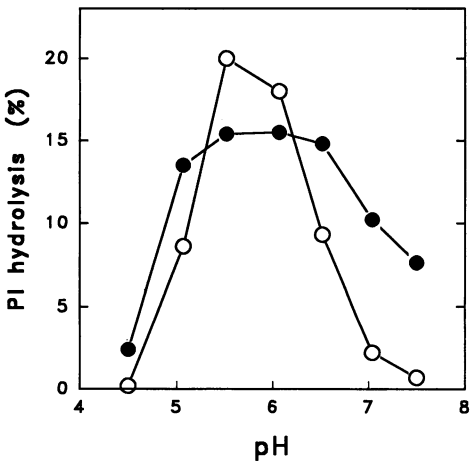


FIG. 4. Effect of pH on PI-PLC activity. [^{3}H]PI suspended in Triton X-100 (0.01% final concentration) was incubated with *S. aureus* PI-PLC purified from *E. coli* transformed with pSF122 (66 ng; ○) or *B. thuringiensis* PI-PLC (0.9 ng; ●) at the pH indicated (100 mM HEPES-MES-NaOH buffer). At hydrolysis values of >30% the assay is nonlinear and underestimates the PI-PLC activity. Values are means from duplicate incubations from a single experiment, one of two experiments giving similar results.

(v) Inhibition by thiol reagents. Previous studies of the PI-PLC from *S. aureus* had indicated that it was sensitive to inhibition by the thiol-reactive agents $HgCl_2$, $CuSO_4$, and *p*-chloromercuribenzoate, suggesting that a cysteine residue might be located in or near the active site (29). However, this possibility was eliminated by the observation that the predicted sequence did not contain any cysteine residues (Fig. 2b). We therefore reevaluated this question using a variety of thiol-reactive reagents assayed under different conditions. Pretreatment with the alkylating agents sodium iodoacetate and *N*-ethylmaleimide (2.5 mM, 60 min, 0°C; data not shown) did not produce significant inhibition of PI degradation by *S. aureus* PI-PLC (from pSF122). Some inhibition was observed with iodoacetate (but not *N*-ethylmaleimide) if the treatment was carried out at 37°C for 30 min, but this amounted to a maximum of only 20 to 30%. *B. thuringiensis* PI-PLC was not inhibited by either of these reagents (data not shown).

In contrast, both the *S. aureus* and the *B. thuringiensis* PI-PLCs were sensitive to inhibition by $HgCl_2$ (50% inhibitory concentration, ~5 to 10 μM) and Cu_2SO_4 (50% inhibitory concentration, ~5 μM for *S. aureus* and ~1 μM for *B. thuringiensis*). Inhibition of PI degradation by these metal ions was similar whether Triton X-100 or deoxycholate was used to suspend the substrate. Inhibition (60 to 90%) was also observed with 0.25 mM *p*-chloromercuriphenylsulfonate. The response to these inhibitors was identical when the two *S. aureus* PI-PLC preparations were compared with each other.

The mechanism of the potent inhibition by heavy metal ions is unclear. The concentrations required were so low (<10 μM) that inhibition is unlikely to have resulted from an effect on substrate presentation due to an interaction between the metal ion and the substrate (100 μM). We propose that inhibition results instead from a direct binding of the metal ion to the PI-PLC via a side-chain group other than cysteine. It is likely that a similar mechanism is responsible for the inhibition of the *B. thuringiensis* PI-PLC because it also is devoid of cysteine residues (Fig. 2b) (14). A cysteine-independent mechanism has been proposed previously for the inhibition of bovine brain PLC-γ by micromolar concentrations of Hg^{2+}, Cu^{2+}, Cd^{2+}, and Zn^{2+} (51).

PI-PLC production is regulated by the *agr* locus. Since the secretion of many *S. aureus* extracellular proteins is known to be regulated by the *agr* locus, it was of interest to determine whether this also applied to PI-PLC. A staphylococcal strain carrying a transposon (Tn*551* which contains an erythromycin resistance gene) insertion in *agr* (RN6112) and its isogenic parent strain (RN6390) were therefore analyzed for their ability to produce PI-PLC. In both CY and THB media the *agr* mutant produced substantially less PI-PLC than the *agr*$^+$ parent strain (Fig. 5). Analysis of the culture supernatants by Western blotting (immunoblotting) with a polyclonal antibody against PI-PLC confirmed that mutation of the *agr* locus reduced the amount of PI-PLC produced (data not shown). However, it is also apparent from Fig. 5 that production of PI-PLC by both the *agr* mutant and the *agr*$^+$ strain was increased three- to sixfold by culturing in THB rather than CY medium. This result suggested that PI-PLC production can be regulated by both *agr*-dependent and *agr*-independent mechanisms. Analysis of the DNA from these strains by Southern blotting indicated that the *plc* locus was identical in both strains and therefore the reduction in PI-PLC activity was not caused by a change in *plc* itself (Fig. 6a, lanes 3 and 4). In addition, RN4256, which has the same Tn*551* insertion in *agr*, shows a similar decrease in

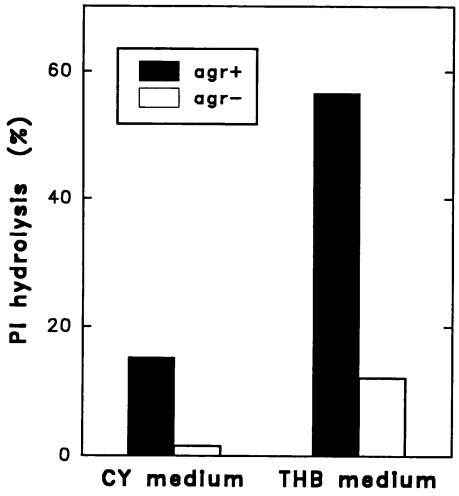


FIG. 5. Regulation of PI-PLC production by the *agr* locus. RN6390 (*agr*$^+$) and RN6112 (*agr* mutant) strains of *S. aureus* were grown overnight in CY medium or THB, and PI-PLC activity in the culture supernatant was determined with PI as a substrate. A similar difference in PI-PLC production was observed when RN6112 was cultured in the presence of erythromycin. There was no obvious difference in the growth rates of the two strains. At hydrolysis values of >30% the assay is nonlinear and underestimates the PI-PLC activity. Values are means from duplicate incubations from a single experiment, one of two giving similar results.

the amount of PI-PLC activity compared with that of its wild-type parent, RN4282 (data not shown). The amount of PI-PLC produced by these strains was also sensitive to the type of medium and increased severalfold when cultured in THB rather than CY medium. However, when analyzed by Southern blotting, the *plc* locus of the parent, RN4282 (Fig. 6a, lane 1), showed a restriction pattern different from that of the mutant RN4256 (Fig. 6a, lane 2) as well as those of RN6390 and RN6112 (Fig. 6a, lanes 3 and 4) and the Newman strain (Fig. 6c, lane 1). Therefore, although the reduced amount of PI-PLC in RN4256 is most likely due to the inactivation of *agr*, this result must be interpreted with caution since the *plc* locus in the mutant is not identical to that of its parent. We also attempted to determine whether the reduced PI-PLC secretion resulted from a decrease in *plc* transcription. However, mRNA encoding PI-PLC was not detectable by Northern blotting in any of these strains (data not shown), and consequently we were unable to confirm that regulation of PI-PLC levels in the culture medium occurred at the transcriptional level. The inability to detect mRNA is consistent with the low level of PI-PLC produced as described below for other *S. aureus* strains.

PI-PLC production by clinical isolates and other *Staphylococcus* species. Preliminary studies of different strains of *S. aureus* had indicated that most produced very little PI-PLC (7). However, the ability of these strains to produce PI-PLC might have been lost during subculture and storage. To address this issue, fresh clinical isolates of *S. aureus* were cultured and the amount of PI-PLC in the supernatant was determined by enzyme assay (Fig. 7). Although all the culture supernatants contained detectable PI-PLC activity there was an ~30-fold range in the amount produced. When these analyses were repeated several months later using frozen stock cultures, most of the strains produced substantially less PI-PLC (data not shown). The maximum amount of PI-PLC produced by the isolates (e.g., C240) was 10 to 20 mU ml^{-1} compared to the 100 to 200 mU ml^{-1} produced by the Newman strain. The culture supernatants were also analyzed by Western blotting with F(ab)$_2$ fragments of a polyclonal antibody raised against purified PI-PLC. Interpretation of these analyses was complicated by cross-reactivity with other proteins in the culture supernatants. In spite of this, the strength of the signal (i.e., a band with mobility similar to that of the purified PI-PLC) correlated reasonably well with the amount of PI-PLC produced (data not shown). mRNA extracted from 6-h cultures of the Newman strain and several of the clinical isolates was visualized by Northern blotting using the *Hin*dIII fragment of pSF122 as a probe. A band of approximately 1 kb was observed in the Newman strain but not in C240, C400, or C389 (data not shown). However, in one RNA preparation a faint 1-kb band was observed with C240. The absence of a positive signal in the Northern blot is consistent with the relatively low levels of PI-PLC produced by the clinical isolates compared with those produced by the Newman strain (Fig. 7).

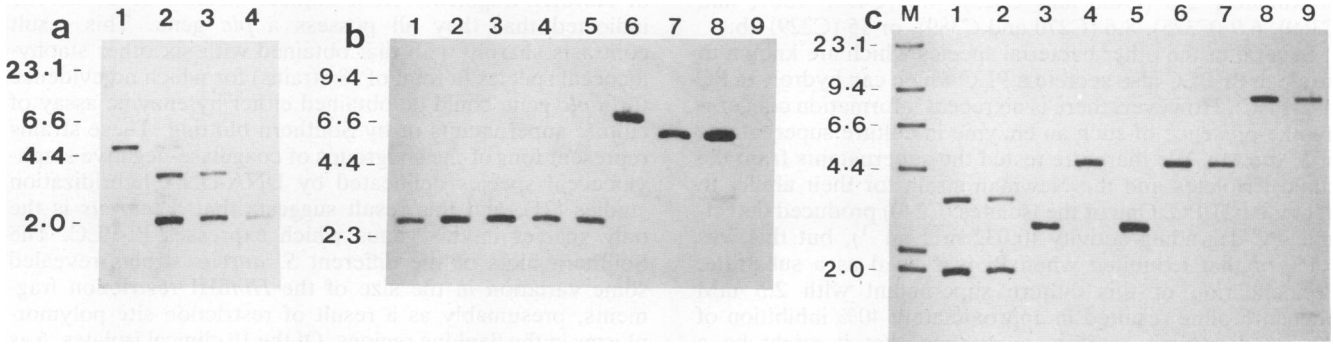


FIG. 6. Southern blotting of the *plc* gene. (a) DNA from *S. aureus*. *agr*$^+$ and *agr* mutant strains was digested with *Sna*BI. Lane 1, RN4282 (*agr*$^+$); lane 2, RN4256 (*agr* mutant); lane 3, RN6390 (*agr*$^+$); lane 4, RN6112 (*agr* mutant). (b) DNA from clinical isolates was digested with *Hin*dIII. Lane 1, Newman; lane 2, C240; lane 3, C490; lane 4, C323; lane 5, C360; lane 6, C325; lane 7, C270; lane 8, C389; lane 9, C572 (*S. epidermidis*). C367, C402, and C229 are not shown but gave single fragments of 2.7, 2.7, and 18 kb, respectively. (c) DNA from clinical isolates digested with *Sna*BI. Lane M, molecular size markers; lane 1, Newman; lane 2, strain C240; lane 3, C400; lane 4, C490; lane 5, C323; lane 6, C367; lane 7, C360; lane 8, C270; lane 9, C389. C367, C325, and C229 are not shown but gave single fragments of 4.6, 6.9, and 15 kb, respectively. Numbers along the left indicate the positions and sizes of markers (in kilobases). The restriction fragments were visualized by a probe consisting of the entire *plc* gene (i.e., a *Hin*dIII fragment of pSF122; Fig. 1).

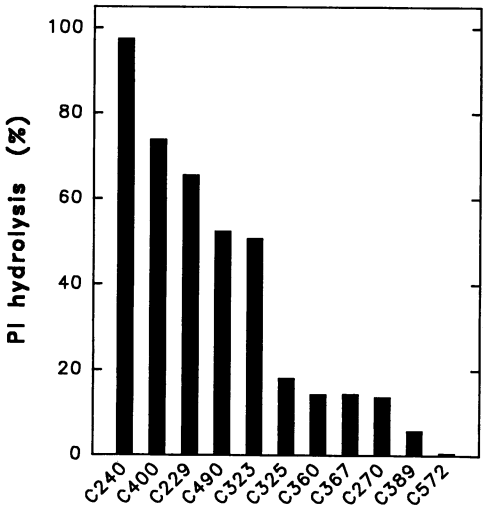


FIG. 7. PI-PLC production by clinical isolates of *S. aureus*. Freshly isolated strains of *S. aureus* (C572 is a clinical isolate of *S. epidermidis*) were cultured in THB, and PI-PLC activity in the supernatant was determined as described in Materials and Methods. Values represent amount of PI hydrolysis given by 0.05 ml of each isolate after 60 min at 37°C (mean from two independent cultures). Values above 30% are underestimates due to nonlinearity of the assay. In other experiments assays of diluted samples indicated that there was a 30-fold difference in activity between C240 and C389. The cultures were also assayed for PC-PLC activity, but only in C240 was significant activity detected (0.032 mU/ml).

When analyzed by Southern blotting with a probe consisting of the entire *plc* gene (Fig. 6b), all 10 isolates gave a positive signal under conditions of high stringency, suggesting that they possess a *plc* gene. However, in four of the strains (C325, C270, C389, and C229) the *Hin*dIII restriction fragment containing *plc* was substantially larger (approximately 6.1, 6.8, or 18 kb) than that observed in the Newman strain (approximately 2.7 kb). The DNA in each isolate was also digested at a site within the *plc* gene with *Sna*BI (Fig. 6c). As expected, Newman gave two fragments of approximately 1.9 and 3.4 kb. However, among the clinical isolates only C240 gave these two fragments (Fig. 6c). In all the other isolates the probe hybridized to only one fragment of approximately 2.8 (C400 and C323), 4.6 (C490, C367, and C360), 6.9 (C325), 8.6 (C270 and C389), or 15 (C229) kb.

Several of the other bacterial species which are known to produce PI-PLC also secrete a PLC which can hydrolyze PC (PC-PLC). However, there is no recent information concerning the presence of such an enzyme in culture supernatants of *S. aureus*. We therefore tested the supernatants from the clinical isolates and the Newman strain for their ability to degrade [^{3}H]PC. One of the isolates (C240) produced detectable PC-degrading activity (0.032 mU ml^{-1}), but this was <1% of that exhibited when PI was used as a substrate. Preincubation of this culture supernatant with 2.5 mM phenanthroline resulted in approximately 40% inhibition of the PC-degrading activity, suggesting that it might be a PC-PLC (24), but the activity was too low for this to be confirmed by product analysis. The other strains did not produce detectable PC-PLC activity (<0.006 mU ml^{-1}).

To determine whether the ability to produce PI-PLC was restricted to *S. aureus*, other staphylococcal species were studied. Culture supernatants of clinical isolates of *S. epidermidis* (a total of five), *S. hominis*, and *Staphylococcus simulans* were tested, but none of them produced detectable PI-PLC activity. Culture supernatants from standard strains of *S. epidermidis* (ATCC 155 and ATCC 14990), *S. hominis* (ATCC 29885), *S. saprophyticus* (ATCC 15305), *S. sciuri* (ATCC 29060), and *S. xylosus* (ATCC 29971) were analyzed also, but no PI-PLC activity could be detected (<0.1 mU/ml; data not shown). *Hin*dIII restriction digests of all 13 of these staphylococcal strains were analyzed by Southern blotting using the *plc* probe, but none gave a positive signal (representative data from an *S. epidermidis* strain, C572, is shown in Fig. 6b and 7).

DISCUSSION

A survey of *S. aureus* isolates from patients with staphylococcal infections showed that approximately 30% produced detectable levels of PI-PLC activity when cultured in vitro whereas *S. aureus* isolated from healthy volunteers did not produce PI-PLC (34). Although this correlation is suggestive of a role for PI-PLC in pathogenesis, there were large variations among the positive strains in the absolute amounts of activity produced. The present study also revealed large variations in PI-PLC production between different isolates when cultured in vitro. In addition, we have previously noticed that PI-PLC production was extremely sensitive to the culture conditions and that the different isolates did not respond uniformly to these variations in the culture conditions (7). These findings make it difficult to extrapolate the results of in vitro studies to the situation in vivo and raise doubts about the significance of any correlations (either positive or negative) between PI-PLC production and staphylococcal disease syndromes. For example, low levels of PI-PLC production could reflect altered sensitivity to multiple and as yet poorly defined regulatory factors or to alterations in the gene encoding PI-PLC. Clearly the contribution of regulatory factors (identified in vitro) to overall PI-PLC production could assume greater or lesser relative importance in vivo.

To gain a better understanding of the regulation of PI-PLC production by *S. aureus* and its possible role in virulence, we have cloned the *plc* gene. This has allowed us to make a preliminary evaluation of the relative contribution of strain variations which affect the *plc* gene as opposed to those which might be affecting regulatory genes. Southern blotting of *Hin*dIII fragments from clinical isolates of *S. aureus* indicated that they all possess a *plc* gene. This result contrasts sharply with that obtained with six other staphylococcal species (a total of 13 strains) for which no evidence for a *plc* gene could be obtained either by enzyme assay of culture supernatants or by Southern blotting. These strains represent four of the six groups of coagulase-negative staphylococcal species delineated by DNA-DNA hybridization studies (21), and this result suggests that *S. aureus* is the only species in this genus which expresses PI-PLC. The Southern blots of the different *S. aureus* strains revealed some variation in the size of the *Hin*dIII restriction fragments, presumably as a result of restriction site polymorphisms in the flanking regions. Of the 10 clinical isolates, 6 as well as the Newman strain yield a 2.7-kb fragment, indicating that the upstream *Hin*dIII site is approximately 1.3 kb from the *plc* gene. To define the variations in flanking sequence more closely, the DNA was digested with *Sna*BI, which cuts in the middle of the *plc* gene. However, these analyses produced an unexpected result. Only 1 (C240) of the 10 isolates gave the two restriction fragments that were

predicted on the basis of sequence analysis of pSF122. This result indicated that the other nine isolates contained a mutation in the coding region of *plc* as well as in flanking sequences. We do not believe that loss (or creation) of *Hin*dIII and *Sna*BI sites in flanking regions would have a direct efffect on the transcription of the *plc* gene. *plc* might be transcribed as part of a polycistronic message originating from a distant promoter site, but this seems unlikely for two reasons. There is no open reading frame immediately upstream, and Northern blotting indicates that the *plc* mRNA in the Newman strain is approximately 1 kb and therefore monocistronic. By contrast, mutation of the internal *Sna*BI site might have resulted in one or more amino acid substitutions which reduced the activity or stability of the secreted PI-PLC. It is interesting to note that the isolate with the highest PI-PLC activity (C240) is the only one to exhibit the same *Sna*BI restriction pattern as the Newman strain.

The production of many extracellular proteins by *S. aureus* has been shown to depend on the action of a common regulatory locus, *agr* (43). It was therefore of interest to determine whether the production of PI-PLC was regulated in a similar fashion. In an isogenic pair of *S. aureus* strains the *agr* mutant strain secreted substantially less PI-PLC than the agr^+ strain. The *agr* locus is known to act as a transcriptional regulator, but we were unable to study this because mRNA encoding PI-PLC could not be detected in either of these strains, presumably because of the low level produced. Although our results indicate that the *agr* locus may play an important role in the regulation of PI-PLC production, it is likely that other factors are involved also. For example, the dependence of PI-PLC production on the type of medium was similar in both the agr^+ and *agr* mutant strains, suggesting that the *agr* locus was not required for the response to this particular set of environmental conditions. Previous studies have demonstrated both *agr*-dependent and *agr*-independent regulation of staphylococcal enterotoxin C production by environmental stimuli (44–46), and the large increases in PI-PLC production observed when *S. aureus* was grown in glucose-free media at low pH (33) suggest *agr*-independent regulation. Consistent with these observations is the recent localization of two pleiotropic mutations, *xpr* (55) and *sar* (6), which affect extracellular protein production, to chromosomal sites distinct from that of *agr*. The isolation of the *plc* gene will undoubtedly facilitate a more detailed evaluation of environmental and genetic factors governing PI-PLC production by *S. aureus*.

One additional factor which might contribute to the variable amount of PI-degrading activity detected in the culture supernatants prepared from the clinical isolates is the production of a distinct phospholipase that was also capable of hydrolyzing PI. The other bacteria which are known to produce PI-PLC also produce a PLC (PC-PLC) with relatively broad substrate specificity which hydrolyzes PC, phosphatidylethanolamine, and phosphatidylserine. Although the ability of this PLC to act on PI was in doubt (11, 24, 57), recent work indicates that the PC-PLCs from *L. monocytogenes* and *B. cereus* do in fact have weak activity against PI (12). *S. aureus* was reported to produce a phospholipase A activity which acted on PC (8, 32), but we are not aware of any recent studies in which culture supernatants have been analyzed for PC-PLC by sensitive assays with radiolabelled substrates. It was therefore a reasonable possibility that the low PI-degrading activity which we detected with some strains of *S. aureus* reflected production of such a PC-PLC. However, our analyses indicate that none of the *S. aureus* strains which exhibit low PI-degrading activity express PC-PLC. Although we cannot exclude the production by *S. aureus* of two distinct PI-degrading activities, the available evidence suggests that it is unlikely.

Marques et al. (33) have reported that recovery of PI-PLC activity from *S. aureus* is markedly enhanced after culture at acidic pH, and they have suggested that this may enhance the contribution of PI-PLC to pathogenesis in abscesses in which the extracellular pH is low. The present studies with the purified enzyme reveal an additional mechanism whereby an acidic environment might enhance the pathogenic contribution of PI-PLC, namely, increased catalytic activity. Acidic environments are also found in phagocytic compartments inside cells, and even though *S. aureus* is not regarded as an intracellular pathogen, it has been reported to persist inside phagocytes or endothelial cells for prolonged periods after uptake (1, 2, 16, 19, 31, 58). Substrates in the lumen of a phagolysosome containing *S. aureus* would be highly susceptible to degradation by PI-PLC because of the close proximity, increased catalytic activity, and increased secretion rate favored by such an environment.

The gram-positive bacteria (i.e., *S. aureus*, *Bacillus anthracis*, *B. cereus*, *B. thuringiensis*, *Clostridium novyi*, and *L. monocytogenes*) which produce PI-PLC are all opportunistic pathogens but differ widely in the types of disease they cause, and consequently it is difficult to envisage PI-PLC playing a similar role in the pathogenic process for each of them. Even so, there is some evidence suggesting that PI-PLC contributes to pathogenesis. For example, studies with *S. aureus* have indicated a correlation between pathogenesis and the ability to produce PI-PLC (34). Furthermore, the present and previous studies indicate that the less pathogenic species of *Staphylococcus* (this paper) and *Listeria* (37) are unable to produce PI-PLC. Studies of virulence in a delta-endotoxin-deficient strain of the insect pathogen *B. thuringiensis* have indicated that production of both the PI-PLC and the PC-PLC is decreased in a spontaneous mutant which is unable to infect insect larvae (64). Finally, site-directed *plc* mutants of *L. monocytogenes* exhibit reduced ability to escape from the phagolysosome after being phagocytosed by macrophages, thereby inhibiting the spread of infection from cell to cell (5, 40). While there are no obvious similarities between the pathogenic mechanisms utilized by *S. aureus*, *L. monocytogenes*, and *B. thuringiensis*, this may simply reflect the fact that there are numerous potential targets for PI-PLC on or in eukaryotic cells. Inositol phospholipids not only are involved in eukaryotic signal transduction processes (47) but also are responsible for membrane anchoring of a large number of cell surface proteins (25). Either of these important functions could be severely compromised by the action of an exogenous bacterial PI-PLC. It is obviously premature to speculate about the identity of molecules in mammalian cells which might be targets for PI-PLC action during a staphylococcal infection. However, loss of important cell surface molecules (25, 26), altered responsiveness to extracellular signals (41, 48, 49, 52, 56), and breakdown of intracellular pools of inositol phospholipids (9) have all been observed in vitro and merit serious consideration.

ACKNOWLEDGMENTS

We thank Pat Andreola for excellent technical assistance. We are also grateful to Howard Goldfine, University of Pennsylvania, for helpful discussions and access to unpublished data.

This work was supported in part by a grant-in-aid from the

American Heart Association (National Center), by NIH grants GM-35873 and GM-35873, and by a gift from Samuel W. Rover.

REFERENCES

1. **Bengualid, V., V. B. Hatcher, B. Diamond, E. A. Blumberg, and F. D. Lowy.** 1990. *Staphylococcus aureus* infection of human endothelial cells potentiates Fc receptor expression. J. Immunol. **145:**4279–4283.
2. **Buisman, H. P., L. F. M. Buys, J. A. M. Langermans, P. J. Van den Broek, and R. Van Furth.** 1991. Effect of probenecid on phagocytosis and intracellular killing of *Staphylococcus aureus* and *Escherichia coli* by human monocytes and granulocytes. Immunology **74:**338–341.
3. **Caird, J. D., and G. M. Wiseman.** 1970. Purification of the delta toxin of *Staphylococcus aureus*. Can. J. Microbiol. **16:**703–708.
4. **Camilli, A., H. Goldfine, and D. A. Portnoy.** 1991. *Listeria monocytogenes* mutants lacking phosphatidylinositol-specific phospholipase C are avirulent. J. Exp. Med. **173:**751–754.
5. **Camilli, A., L. G. Tilney, and D. A. Portnoy.** 1993. Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. Mol. Microbiol. **8:**143–157.
6. **Cheung, A. L., J. M. Koomey, C. A. Butler, S. J. Projan, and V. A. Fischetti.** 1992. Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. Proc. Natl. Acad. Sci. USA **89:**6462–6466.
7. **Daugherty, S., and M. G. Low.** 1993. Unpublished data.
8. **Doery, H. M., B. J. Magnusson, J. Gulasekharam, and J. E. Pearson.** 1965. The properties of phospholipase enzymes in staphylococcal toxins. J. Gen. Microbiol. **40:**283–296.
9. **Eberhard, D. A., C. L. Cooper, M. G. Low, and R. W. Holz.** 1990. Evidence that the inositol phospholipids are necessary for exocytosis. Loss of inositol phospholipids and inhibition of secretion in permeabilized cells caused by a bacterial phospholipase C and removal of ATP. Biochem. J. **268:**15–25.
10. **Ferguson, M. A. J., M. G. Low, and G. A. M. Cross.** 1985. Glycosyl-*sn*-1,2-dimyristylphosphatidylinositol is covalently linked to *Trypanosoma brucei* variant surface glycoprotein. J. Biol. Chem. **260:**14547–14555.
11. **Geoffroy, C., J. Raveneau, J.-L. Beretti, A. Lecroisey, J.-A. Vazquez-Boland, J. E. Alouf, and P. Berche.** 1991. Purification and characterization of an extracellular 29-kilodalton phospholipase C from *Listeria monocytogenes*. Infect. Immun. **59:**2382–2388.
12. **Goldfine, H., N. C. Johnston, and C. Knob.** 1993. Nonspecific phospholipase C of *Listeria monocytogenes*: activity on phospholipids in Triton X-100 mixed micelles and in biological membranes. J. Bacteriol. **175:**4298–4306.
13. **Goldfine, H., and C. Knob.** 1992. Purification and characterization of *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C. Infect. Immun. **60:**4059–4067.
14. **Griffith, O. H., J. J. Volwerk, and A. Kuppe.** 1991. Phosphatidylinositol-specific phospholipases C from *Bacillus cereus* and *Bacillus thuringiensis*. Methods Enzymol. **197:**493–502.
15. **Guddal, P. H., T. Johansen, K. Schulstad, and C. Little.** 1989. Apparent phosphate retrieval system in *Bacillus cereus*. J. Bacteriol. **171:**5702–5706.
16. **Hamill, R. J., J. M. Vann, and R. A. Proctor.** 1986. Phagocytosis of *Staphylococcus aureus* by cultured bovine aortic endothelial cells: model for postadherence events in endovascular infections. Infect. Immun. **54:**833–836.
17. **Henner, D. J., M. Yang, E. Chen, R. Hellmiss, H. Rodriguez, and M. G. Low.** 1988. Sequence of the *Bacillus thuringiensis* phosphatidylinositol specific phospholipase C. Nucleic Acids Res. **16:**10383.
18. **Hereld, D., G. W. Hart, and P. T. Englund.** 1988. cDNA encoding the glycosyl-phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei*. Proc. Natl. Acad. Sci. USA **85:**8914–8918.
19. **Hiemstra, P. S., A. Annema, E. F. Schippers, and R. Van Furth.** 1992. Pertussis toxin partially inhibits phagocytosis of immunoglobulin G-opsonized *Staphylococcus aureus* by human granulocytes but does not affect intracellular killing. Infect. Immun. **60:**202–205.
20. **Huang, K.-S., S. Li, and M. G. Low.** 1991. Glycosylphosphatidylinositol-specific phospholipase D. Methods Enzymol. **197:** 567–575.
21. **Kloos, W. E.** 1980. Natural populations of the genus *Staphylococcus*. Annu. Rev. Microbiol. **34:**559–592.
22. **Kuppe, A., L. M. Evans, D. A. McMillen, and O. H. Griffith.** 1989. Phosphatidylinositol-specific phospholipase C of *Bacillus cereus*: cloning, sequencing, and relationship to other phospholipases. J. Bacteriol. **171:**6077–6083.
23. **Leimeister-Wachter, M., E. Domann, and T. Chakraborty.** 1991. Detection of a gene encoding a phosphatidylinositol-specific phospholipase C that is co-ordinately regulated with listeriolysin in *Listeria monocytogenes*. Mol. Microbiol. **5:**361–366.
24. **Little, C.** 1981. Phospholipase C from *Bacillus cereus*. Methods Enzymol. **71:**725–730.
25. **Low, M. G.** 1989. The glycosyl-phosphatidylinositol anchor of membrane proteins. Biochim. Biophys. Acta **988:**427–454.
26. **Low, M. G.** 1990. Degradation of glycosyl-phosphatidylinositol anchors by specific phospholipases, p. 35–63. *In* A. J. Turner, (ed.), Cell and molecular biology of membrane proteins. Ellis Horwood, Chichester, United Kingdom.
27. **Low, M. G.** 1992. Phospholipases that degrade the glycosyl-phosphatidylinositol anchor of membrane proteins, p. 117–154. *In* N. M. Hooper and A. J. Turner, (ed.), Lipid modification of proteins: a practical approach. Oxford University Press, Oxford.
28. **Low, M. G.** 1993. Upublished data.
29. **Low, M. G., and J. B. Finean.** 1977. Modification of erythrocyte membranes by a purified phosphatidylinositol-specific phospholipase C (*Staphylococcus aureus*). Biochem. J. **162:**235–240.
30. **Low, M. G., J. Stiernberg, G. L. Waneck, R. A. Flavell, and P. W. Kincade.** 1988. Cell-specific heterogeneity in sensitivity of phosphatidylinositol-anchored membrane antigens to release by phospholipase C. J. Immunol. Methods **113:**101–111.
31. **Lowy, F. D., J. Fant, L. L. Higgins, S. K. Ogawa, and V. B. Hatcher.** 1988. *Staphylococcus aureus*-human endothelial cell interactions. J. Ultrastruct. Mol. Struct. Res. **98:**137–146.
32. **Magnusson, B. J., H. M. Doery, and J. Gulasekharam.** 1962. Phospholipase activity of staphylococcal toxin. Nature (London) **196:**270–271.
33. **Marques, M. B., P. F. Weller, and A. Nicholson-Weller.** 1992. Growth in acidic media increases production of phosphatidylinositol-specific phospholipase C by *Staphylococcus aureus*. Curr. Microbiol. **25:**125–128.
34. **Marques, M. B., P. F. Weller, J. Parsonnet, B. J. Ransil, and A. Nicholson-Weller.** 1989. Phosphatidylinositol-specific phospholipase C, a possible virulence factor of *Staphylococcus aureus*. J. Clin. Microbiol. **27:**2451–2454.
35. **Mengaud, J., C. Braun-Breton, and P. Cossart.** 1991. Identification of phosphatidylinositol-specific phospholipase C activity in *Listeria monocytogenes*: a novel type of virulence factor? Mol. Microbiol. **5:**367–372.
36. **Nesin, M., P. Svec, J. R. Lupski, G. N. Godson, B. Kreiswirth, J. Kornblum, and S. J. Projan.** 1990. Cloning and nucleotide sequence of a chromosomally encoded tetracycline resistance determinant, *tet4*(M), from a pathogenic, methicillin-resistant strain of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **34:**2273–2276.
37. **Notermans, S., J. Dufrenne, M. Leimeister-Wächter, E. Domann, and T. Chakraborty.** 1991. Phosphatidylinositol-specific phospholipase C activity as a marker to distinguish between pathogenic and nonpathogenic *Listeria* species. Appl. Environ. Microbiol. **57:**2666–2670.
38. **Peng, H.-L., R. P. Novick, B. Kreiswirth, J. Kornblum, and P. Schlievert.** 1988. Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. J. Bacteriol. **170:**4365–4372.
39. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guandinium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
40. **Portnoy, D. A., T. Chakraborty, W. Goebel, and P. Cossart.**

1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. **60**:1263–1267.

41. **Presky, D. H., M. G. Low, and E. M. Shevach.** 1990. Role of phosphatidylinositol-anchored proteins in T cell activation. J. Immunol. **144**:860–868.
42. **Projan, S. J., J. Kornblum, B. Kreiswirth, S. L. Moghazeh, W. Eisner, and R. P. Novick.** 1989. Nucleotide sequence of the β-hemolysin gene of *Staphylococcus aureus*. Nucleic Acids Res. **17**:3305.
43. **Recsei, R. P., B. Kreiswirth, M. O'Reilly, P. Schlievert, A. Gruss, and R. P. Novick.** 1986. Regulation of exoprotein gene expression in *Staphylococcus aureus* by *agr*. Mol. Gen. Genet. **202**:58–61.
44. **Regassa, L. B., and M. J. Betley.** 1992. Alkaline pH decreases expression of the accessory gene regulator (*agr*) in *Staphylococcus aureus*. J. Bacteriol. **174**:5095–5100.
45. **Regassa, L. B., and M. J. Betley.** 1993. High sodium chloride concentrations inhibit staphylococcal enterotoxin C gene (*sec*) expression at the level of *sec* mRNA. Infect. Immun. **61**:1581–1585.
46. **Regassa, L. B., R. P. Novick, and M. J. Betley.** 1992. Glucose and nonmaintained pH decrease expression of the accessory gene regulator (*agr*) in *Staphylococcus aureus*. Infect. Immun. **60**:3381–3388.
47. **Rhee, S. G., and K. D. Choi.** 1992. Regulation of inositol phospholipid-specific phospholipase C isozymes. J. Biol. Chem. **267**:12393–12396.
48. **Robinson, P. J.** 1991. Signal transduction by GPI-anchored membrane proteins. Cell Biol. Int. Rep. **15**:761–767.
49. **Robinson, P. J., and S. C. Spencer.** 1988. Phosphatidylinositol-anchored membrane proteins and lymphocyte activation. Immunol. Lett. **19**:85–94.
50. **Ross, T. S., and P. W. Majerus.** 1986. Isolation of D-*myo*-inositol 1:2-cyclic phosphate 2-inositolphosphohydrolase from human placenta. J. Biol. Chem. **261**:11119–11123.
51. **Ryu, S. H., K. S. Cho, K.-Y. Lee, P.-G. Suh, and S. G. Rhee.** 1987. Purification and characterization of two immunologically distinct phosphoinositide-specific phospholipase C from bovine brain. J. Biol. Chem. **262**:12511–12518.
52. **Saltiel, A. R., and L. R. Sorbara-Cazan.** 1987. Inositol glycan mimics the action of insulin on glucose utilization in rat adipocytes. Biochem. Biophys. Res. Commun. **149**:1084–1092.
53. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
54. **Sandler, P., and B. Weisblum.** 1988. Erythromycin-induced stabilization of *ermA* messenger RNA in *Staphylococcus aureus* and *Bacillus subtilis*. J. Mol. Biol. **203**:905–915.
55. **Smeltzer, M. S., M. E. Hart, and J. J. Iandolo.** 1993. Phenotypic characterization of *xpr*, a global regulator of extracellular virulence factors in *Staphylococcus aureus*. Infect. Immun. **61**:919–925.
56. **Stiernberg, J., M. G. Low, L. Flaherty, and P. W. Kincade.** 1987. Removal of lymphocyte surface molecules with phosphatidylinositol-specific phopholipase C: effects on mitogen responses and evidence that ThB and certain Qa antigens are membrane-anchored via phosphatidylinositol. J. Immunol. **38**: 3877–3884.
57. **Titball, R. W.** 1993. Bacterial phospholipases C. Microbiol. Rev. **57**:347–366.
58. **Vann, J. M., and R. A. Proctor.** 1987. Ingestion of *Staphylococcus aureus* by bovine endothelial cells results in time- and inoculum-dependent damage to endothelial cell monolayers. Infect. Immun. **55**:2155–2163.
59. **Vazquez-Boland, J.-A., C. Kocks, S. Dramsi, H. Ohayon, C. Geoffroy, J. Mengaud, and P. Cossart.** 1992. Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. Infect. Immun. **60**:219–230.
60. **Volwerk, J. J., M. S. Shashidhar, A. Kuppe, and O. H. Griffith.** 1990. Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* combines intrinsic phosphotransferase and cyclic phosphodiesterase activities: ^{31}P NMR study. Biochemistry **29**:8056–8062.
61. **von Heijne, G.** 1985. Signal sequences: the limits of variation. J. Mol. Biol. **189**:239–242.
62. **Wiseman, G. M.** 1975. The hemolysins of *Staphylococcus aureus*. Bacteriol. Rev. **39**:317–344.
63. **Wiseman, G. M., and J. D. Caird.** 1968. Phospholipase activity of the delta hemolysin of *Staphylococcus aureus*. Proc. Soc. Exp. Biol. Med. **128**:428–430.
64. **Zhang, M.-Y., A. Lovgren, M. G. Low, and R. Landen.** 1993. Characterization of an avirulent pleiotropic mutant of the insect pathogen *Bacillus thuringiensis:* reduced expression of flagellin and phospholipases. Infect. Immun. **61**:4947–4954.