The *Arcanobacterium* (*Actinomyces*) *pyogenes* Hemolysin, Pyolysin, Is a Novel Member of the Thiol-Activated Cytolysin Family

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*Arcanobacterium* (*Actinomyces*) *pyogenes*, an animal pathogen, produces a hemolytic exotoxin, pyolysin (PLO). The gene encoding PLO was cloned, and sequence analysis revealed an open reading frame of 1,605 bp encoding a protein of 57.9 kDa. PLO has 30 to 40% identity with the thiol-activated cytolsins (TACYs) of a number of gram-positive bacteria. The activity of PLO was found to be very similar to those of other TACYs, except that it was not thiol activated. The highly conserved TACY undecapeptide is divergent in PLO; in particular, the cysteine residue required for thiol activation has been replaced with alanine. However, mutagenesis of the alanine residue to cysteine did not confer thiol activation on PLO, suggesting a conformational difference in the undecapeptide region of this toxin. Specific antibodies against purified, recombinant PLO completely neutralized the hemolytic activity of *A. pyogenes*, suggesting that this organism produces a single hemolysin. Furthermore, these antibodies could passively protect mice against lethal challenge with *A. pyogenes*, suggesting that like other TACYs PLO is an important virulence factor in the pathogenesis of this organism.

*Arcanobacterium* (*Actinomyces*) *pyogenes* (36), a gram-positive, normally commensal bacterium, resides on the mucous membranes of cattle, sheep, swine, and other economically important animals (10). It can, through an as yet unknown mechanism, disseminate to cause a wide variety of nonspecific purulent infections involving the visceral organs (19, 25, 45) and joints (18), as well as acute purulent mastitis (20), chronic purulent infections involving the visceral organs (19, 25, 45) and joints (18), as well as acute purulent mastitis (20), chronic abscessing mastitis (37), and abortion (42).

Despite the versatility of *A. pyogenes* as an agent of disease in domestic animals, specific determinants of its virulence have not been characterized. *A. pyogenes* produces several potential virulence factors including a DNase (24) and several proteases (40, 44). In addition to these factors, *A. pyogenes* produces hemolytic exotoxin pyolysin (PLO) (13), which is cytolytic for the erythrocytes of a number of animal species (14), as well as dermonecrotic and lethal for laboratory animals (27). PLO also exhibits cytotoxic effects on bovine polymorphonuclear leukocytes (PMN) and kangaroo kidney cells (13). PLO was reported to be oxygen stable, and its activity was reported to be unaffected by cholesterol (14). The role of this toxin in pathogenesis is unclear. However, it is expressed in vivo and is immunogenic, as antihemolysin antibodies have been found in unaffected animals (8, 12), and its potential role in virulence.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Escherichia coli DH5α (Bethesda Research Laboratories [BRL]), DH5α F’<ac’> (BRL), and LE392 (Promega) were grown on either Luria-Bertani (LB) agar or in LB broth (Difco) at 37°C. Antibiotics (Sigma) were added as appropriate at the following concentrations: ampicillin, 100 μg/ml; and tetracycline, 15 μg/ml. *A. pyogenes* BBRI (Marana, Ariz.), OX1 and OX8 (Oxford Laboratories, Worthington, Mnn.), and ATCC 19411 (American Type Culture Collection) were grown on brain heart infusion (BHI) agar (Difco) supplemented with 5% bovine blood at 37°C and 5% CO2 in a humidified incubator. Liquid cultures of *A. pyogenes* were grown in BHI broth supplemented with 5% fetal bovine serum at 37°C. Additional *A. pyogenes* strains were obtained from veterinary diagnostic laboratories or personal culture collections. *Listeria monocytogenes* 3a Xb (Centers for Disease Control and Prevention, Atlanta, Ga.) was grown on BHI agar plates or in BHI broth.

**Preparation of native PLO or LLO.** Cultures of *A. pyogenes* or *L. monocytogenes* were grown to an optical density at 600 nm of approximately 3.0 to 4.0. Culture supernatant fluid (CSF) was obtained by passage of the culture through a 0.22-μm-pore-size filter and was used as a source of native PLO or listeriolysin O (LLO). Where necessary, CSF was concentrated by ultrafiltration with a YM30 membrane (Amicon).

**DNA techniques.** Procedures for *E. coli* transformation and plasmid extraction, DNA restriction, ligation, agarose gel electrophoresis, and Southern blotting were performed as described previously (3). Preparation of DNA probes, DNA hybridization, and probe detection were performed with the DIG DNA labeling and detection kit (Boehringer Mannheim). PCR DNA amplification was performed with *Tag* DNA polymerase (Fisher Scientific) for 35 cycles consisting of 1 min at 94°C (DNA denaturation), 1 min at 55°C (primer annealing), and 1 min at 72°C (DNA synthesis).

**Cloning of the *A. pyogenes* plo gene.** Genomic DNA was prepared from *A. pyogenes* BBRI as previously described (43). A library of partially RamHI-digested genomic DNA from BBRI was prepared in the cosmid vector pLAFR2 (Cold Spring Harbor Laboratories). The ligation mixture was packaged with the Packagene system according to the manufacturer’s instructions (Promega). *E. coli* LE392 was transduced to Tc resistance, and the hemolytic transductants were selected on LB agar with 5% ovine blood and 15 μg of tetracycline per ml. Two hemolytic colonies containing cosmids ApH1 and ApH2 were selected. A 3.5-kb XhoI-EcoRI fragment from ApH1 containing the plo gene was blunted at the EcoRI site with T4 DNA polymerase and cloned into XhoI-HindIII-digested pBluescript SKI (+) (Stratagene) to create pApS50.

**Nucleotide sequence determination.** Appropriate subclones and nested deletions, prepared with Exonuclease III/Mung Bean Nuclease (Ambion) as de-
scribed previously (3), were sequenced by the dideoxy chain termination method (38) with Sequenase (U.S. Biochemicals). Sequencing was performed on both strands, across all restriction sites. DNA sequence data were confirmed by automated sequencing on a 373A DNA sequencer (Applied Biosystems, Inc.).

Cloning and purification of recombinant His-PLO. The plo gene, lacking the coding region for the predicted signal sequence, was amplified from A. pyogenes BBRI genomic DNA by PCR with a 5′ primer containing an Xhol site (5′-ACGACATGCTGGCGGATTGGAAC-3′) and a 3′ primer containing an EcoRI site (5′-GGATCCTGGAATATCGGATTGGAAC-3′). A 1.5-kb PCR fragment was digested with Xhol-EcoRI and cloned into XhoI-EcoRI-digested pTrcHis B (Invitrogen) to generate pGS59. The entire insert of pGS59 was sequenced to confirm that no mutations were introduced by PCR. Cultures for the preparation of His-tagged PLO (His-PLO) or His-PLO.C492 were grown to an optical density at 600 nm of 0.6 prior to induction with 5 μg isopropyl-β-D-thiogalactopyranoside (IPTG; Gold Biotechnology) for 3 h. Cells were harvested by centrifugation at 5,000 × g, and the cell pellet was resuspended in phosphate-buffered saline (PBS; 0.01 M; pH 7.2). The cells were disrupted by passage through a 3-cm3 syringe. Serial dilutions obtained by lavage with 3 ml of PBS. Blood and liver were aseptically removed, on days 4, 7, and 13 postchallenge or when moribund. Peritoneal fluid (PF) was obtained 8.5 h after the last immunization (day 0). Mice were euthanized by cervical dislocation.

Preparation of goat antiserum to His-PLO (α-His-PLO). An adult female goat was immunized with 500 μg of His-PLO in Ribi Adjuvant System (Ribi ImmunoChem Research Inc.) administered intramuscularly in the hind leg at two week intervals. A booster injection of 600 μg of His-PLO in Ribi Adjuvant System was administered 25 days later. Blood was collected on day 46, and antiserum was harvested from the clotted blood. Preimmune serum was collected in a similar manner prior to immunization.

Passive immunization of mice with α-His-PLO. Groups of six female, 6- to 8-week-old ICR mice (Harlan Sprague Dawley) were immunized with similar booster injection of 600 μg of His-PLO. The System was administered 25 days later. Blood was collected on day 46, and antiserum was harvested from the clotted blood. Preimmune serum was collected in a similar manner prior to immunization.

Hemolytic assays. Sera from infected mice were serially diluted, 10-fold, into 1/2% washed sheep erythrocyte suspensions, to final concentrations of 10-4, 10-5, or 10-6 HU/ml in PBS. After a 30-min incubation at 37°C, 100 μl of dilutions and 100 μl of PBS were added to 1-ml aliquots of 1,280-HU/ml hemolysin preparations, to final concentrations of 1,280-HU/ml hemolysin preparations with either 2.5 mM dithiothreitol (DTT), 2% β-mercaptoethanol (β-ME), or 500 μM 5,5′-dithiobis(2-nitro-benzonic acid) (DTNB) (all concentrations are final) for 10 min or with 1 mM (final concentration) N-ethylmaleimide (MalNE) for 30 min at room temperature for hemolytic assay. To assay for cholesterol inhibition, 100 μM cholesterol in absolute ethanol were added to 1-ml aliquots of 1,280-HU/ml hemolysin preparations, to final concentrations of 1 μM/ml or 100 ng/ml. After a 30-min incubation at room temperature, the hemolytic assay was performed as described above, with hemolysin preparations incubated with 100 μl of absolute ethanol as controls.

The hemolytic titers of sera were assessed by incubating 5 IU of PLO with serial twofold dilutions of sera at 37°C for 30 min prior to assaying for hemolytic activity as described above. The antigenic titer was the reciprocal of the antibody titer which completely neutralized hemolytic activity.

SDS-PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed essentially as described by Khadik et al. (6), except that a final concentration of 0.25% w/v sodium dodecyl sulfate (SDS) was used. One hundred micrograms of His-PLO protein was used in each lane of the gel. Unstained bands were visualized using Commassie blue (Bio-Rad) followed by autoradiography (12). The primary antibody used was goat antiserum to His-PLO (α-His-PLO).

Nucleotide sequence accession number. Nucleotide sequence data reported in this study were deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number U84782.

RESULTS

Cloning and expression of the A. pyogenes plo gene. The plo gene encoding the hemolysin, PLO, of A. pyogenes BBRI was cloned in E. coli with selection for hemolytic activity on blood agar as described in Materials and Methods. Two hemolytic colonies were observed, and the resulting cosmids, ApH1 and ApH2, overlapped significantly in a restriction endonuclease profile. Successive subcloning from ApH1 yielded plasmid pAP350, which contained a 3.5-kb EcoRI-XhoI fragment (Fig. 1). pAP350 was still able to confer hemolytic activity on E. coli DH5α, but all attempts to reduce the size of pAP350 resulted in nonhemolytic recombinants. An identically sized 3.5-kb EcoRI-XhoI fragment was detected in BBRI genomic DNA by Southern blotting with a digoxigenin-labeled probe derived from the insert of pAP350 (data not shown).

Nucleotide sequence of the A. pyogenes plo gene. Nucleotide sequence data obtained from pAP350 revealed an open reading frame (ORF) of 1,605 bp (Fig. 2). Deletions extending into this ORF yielded nonhemolytic colonies, suggesting that this ORF encoded an A. pyogenes hemolysin. The gene containing the ORF was designated as plo. The plo gene was preceded by a consensus ribosome binding site and a region of dyad symmetry which may act as a restriction endonuclease recognition site. The ORF encoded a protein of 534 amino acids (aa) in length, with a deduced molecular mass of 57.9 kDa. This presumably represents a putative membrane protein, with eight putative transmembrane domains, and possesses residues generally associated with these structures (Fig. 2).

Searches of the protein databases indicated that the translated amino acid sequence of the encoded protein contains homology with the E. coli plo gene. The A. pyogenes plo gene was subjected to PCR analysis using primers designed to amplify a 455-bp fragment internal to the plo gene. All isolates produced identical bands of the appropriate size (data not shown), suggesting that the gene encoding PLO is present in most, if not all, A. pyogenes isolates.

Analysis of the primary structure of PLO. The predicted PLO protein is 534 amino acids (aa) in length, with a deduced molecular mass of 57.9 kDa. This presumably represents a...
conserved Tyr and Trp residues are shown in boldface. The conserved undecapeptide sequence and ribosome binding site (rbs) are indicated. Arrows are used to denote repeat sequences, and divergent arrows indicate the putative cleavage site between aa 27 and 28 of the protein precursor form of the protein, as there is a consensus signal peptidase cleavage site near the C terminus of the protein. While much of this sequence is also conserved in the majority of sequences are boxed. The Cys residues within the undecapeptides of TACYs are conserved at high frequencies with respect to their frequencies within the PLO molecule (Fig. 2). Three of the six conserved Trp residues are in the undecapeptide sequence (Fig. 3A), while two are contained within a domain implicated in the antibody-independent activation of complement by pneumolysin (PLY) (31). The amino acids demonstrated to be involved in this activity are also conserved in PLO (Fig. 3B).

Phylogenetic comparisons of members of the TACY family indicated that while PLO is clearly a member of this family, it is the most divergent member (Fig. 4). Its closest relatives within the family are the cytolsins of Listeria spp., but in general terms it showed a similar level of identity (30 to 40%) with all members of the family.

Construction and expression of a His-pla gene fusion. To facilitate the purification of recombinant PLO from E. coli, the PLO-coding sequence, lacking the coding sequence for the putative signal peptide, was cloned into pTrcHis B. This construct, pJGS59 (Fig. 1), encoded a 543-aa protein composed of 507 aa of the mature PLO with an N-terminal extension of 36 aa encoded by pTrcHis B, including a six-HIS sequence. SDS-PAGE analysis of IPTG-induced cultures of DH5α-His-PLO (pJGS59) indicated the presence of an overexpressed protein of approximately 59 kDa which was not present in induced cultures of DH5α-F lacF (pJGS59) indicated the presence of an overexpressed protein of approximately 59 kDa which was not present in induced cultures of DH5α-F lacF (pTrcHis B) (Fig. 5A). The size of this protein corresponded to that predicted for the N-terminal His-tagged PLO molecule (His-PLO). DH5α-F lacF (pJGS59) was hemolytic on LB agar supplemented with 5% bovine blood, indicating that His-PLO retained hemolytic activity. His-PLO was purified to near homogeneity (Fig. 5A) and retained hemolytic activity in purified form.

Recognition and inhibition of PLO by antibodies to His-PLO. Goat antiserum prepared against His-PLO (α-His-PLO)
lower-molecular-weight reactive bands may represent degradation products of PLO, as concentrated supernatants from stationary-phase cultures have significant levels of protease activity (data not shown).

**PLO activity is insensitive to reducing agents.** Funk et al. (14) reported that the activity of *A. pyogenes* hemolysin was unaffected by reducing compounds. To confirm this observation, crude PLO and crude and purified His-PLO were preincubated with either β-ME or DTT prior to being assayed for hemolytic activity. In each case there was no effect of reducing agents on hemolytic titer compared to a control preparation of LLO, which exhibited at least a 16-fold increase in hemolytic titer in the presence of reducing agents (Table 1).

To confirm whether the lack of the conserved Cys in the undecapeptide region was responsible for the oxygen-insensitive, or non-thiol-activated, nature of PLO, site-directed mutagenesis was performed on pJGS59 to change the Ala residue (A492) at position 2 of the undecapeptide to Cys. The resulting plasmid was designated pJGS89. In crude extracts, the modified His-PLO molecule (His-PLO.C492), like its parent, showed no increase in hemolytic titer in the presence of reducing agents (Table 1). To confirm that modification of the free thiol group of His-PLO.C492 did not affect hemolytic activity, assays were repeated with purified His-PLO.C492 in the presence of DTNB or MalNEt. The activity of His-PLO.C492, like its parent, showed no increase in hemolytic titer in the presence of reducing agents (Table 1).

**PLO activity is sensitive to cholesterol.** Differences between PLO and other members of the TACY family in the undecapeptide region led us to speculate that PLO may interact differently with cholesterol in the cell membrane. The ability of free cholesterol to inhibit the hemolytic activity of PLO or His-PLO was compared with its ability to inhibit thiol-activated LLO. The activity of each hemolysin was inhibited 75% by incubation with 1 μg of free cholesterol per ml, but no inhibition was observed in the presence of 100 ng of free cholesterol per ml (Table 1). This result is in contrast to that of a previous study in which the activity of *A. pyogenes* hemolysin was reported to be insensitive to cholesterol at a concentration of 1 μg/ml (14).

**Passive protection of mice with α-His-PLO.** The lethality of PLO to mice precluded active immunization with this toxin (27), and passive immunization was undertaken as an alterna-

<table>
<thead>
<tr>
<th>TABLE 1. Effect of reducing agents, thiol-reactive compounds, and cholesterol on hemolytic activity</th>
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<tbody>
<tr>
<td>Hemolysin</td>
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<td></td>
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<tr>
<td>Crude PLO</td>
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<tr>
<td>Crude His-PLO</td>
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<tr>
<td>Crude His-PLO.C492</td>
</tr>
<tr>
<td>Crude LLO</td>
</tr>
<tr>
<td>His-PLO</td>
</tr>
<tr>
<td>His-PLO.C492</td>
</tr>
<tr>
<td>PFO</td>
</tr>
</tbody>
</table>

*p ND, not determined.*

*Crude preparations of His-PLO and His-PLO.C492 were soluble cell fractions from DH5αlacF' (pBrGS59 and pJGS89, respectively).

*LLO preparations were stored at 4°C for >2 weeks and had lost all detectable activity in the absence of reducing agents.

*Hemolytic assay performed in the presence of 2% β-ME.*
the undecapeptide, it possesses significant changes in what is vir-
while PLO has sequence similarity to the conserved TACY
activation does not clearly define this group of toxins. Second,
because it has been demonstrated that some TACYs do not
is clearly the most divergent member of the family. In addition,
PLO is deficient in two of the defining characteristics of this
organism. Despite its clear relationship with the TACYs, PLO
is the most divergent member of the family. In addition,
organism. Because it has been demonstrated that some TACYs do not
exhibit this function in purified form (1, 21, 39), perhaps thiol
activation does not clearly define this group of toxins. Second,
while PLO has sequence similarity to the conserved TACY
undecapeptide, it possesses significant changes in what is vir-
tively. α-His-PLO, but not preimmune serum or PBS,
passively protected mice against A. pyogenes BBR1 infection.
Mice receiving α-His-PLO showed no sign of illness, nor could
any viable bacteria be recovered from blood, PF, or liver. In
contrast, all mice receiving only PBS were dead or moribund by
day 5, with average bacterial counts of 9.1 × 10^9 CFU/ml in
blood, 8.8 × 10^6 CFU/ml in PF, and 1.9 × 10^11 CFU/g in liver
(Table 2). Diffuquik-stained smears of PF indicated that A.
pyogenes BBR1 was readily phagocyted by peritoneal mac-
rophages and PMN. However, a significant proportion of the
bacteria were associated with lysed phagocytes (data not
shown), suggesting that A. pyogenes may kill, rather than be
killed by, these cells. Five of six mice which received preim-
mune serum showed signs of illness, with average bacterial
counts of 1.4 × 10^9 CFU/ml in blood, 6.6 × 10^8 CFU/ml in PF,
and 7.0 × 10^6 CFU/g in liver (Table 2). Three of these mice
were moribund by day 11. It should be noted that the preim-
mune serum had an antihemolytic titer of 4, compared with
1,024 for α-His-PLO. Low levels of antibodies to PLO are
difficult to avoid due to the prevalence of A. pyogenes in adult
animals (26). These low levels of antibodies may have given
rise to the increased survival times observed for mice immu-
nized with preimmune serum compared with those for the
PBS-immunized group. However, there were significant differ-
ences in signs of illness and bacterial viable counts between
these two control groups and the group immunized with α-His-
PLO.

**DISCUSSION**

We have demonstrated that the A. pyogenes hemolytic exo-
toxin, PLO, is a member of the TACY family of pore-forming
 toxins and is probably the sole hemolysin produced by this
organism. Despite its clear relationship with the TACYs, PLO
is clearly the most divergent member of the family. In addition,
PLO is deficient in two of the defining characteristics of this
group of proteins. First, its hemolytic activity is not thiol acti-
ated, a characteristic for which the group is named. However,
because it has been demonstrated that some TACYs do not
exhibit this function in purified form (1, 21, 39), perhaps thiol
activation does not clearly define this group of toxins. Second,
while PLO has sequence similarity to the conserved TACY
undecapeptide, it possesses significant changes in what is vir-
tually an invariant sequence in all other TACYs. It is the first
member of this family to be identified with Ala instead of Cys
at position 2 of the undecapeptide, although Cys-to-Ala mu-
tations at this position in PLY, streptolysin O (SLO), and LLO
result in oxygen-stable molecules (30, 35, 39). It is possible that
thiol activation of TACYs regulates cytolytic activity in vivo,
although, at least in the case of LLO, the Cys-to-Ala mutation
results in LLO which exhibits no significant difference in viru-
ulence from wild-type LLO in a mouse model (30).

It seemed logical to speculate that the lack of the conserved
Cys in the undecapeptide of PLO was the sole reason that PLO
did not exhibit thiol activation. However, clearly this is not the
case, as His-PLO.C492 is not activated in the presence of re-
ducing agents. The PLO undecapeptide has other changes with
respect to the conserved sequence. Insertion of a Pro residue
and deletion of the terminal conserved Arg residue are likely
to have significant effects on both the conformation and charge
of the undecapeptide region. Indeed, insertion of the Pro res-
idue is likely to disrupt the potentially amphiphilic helical
structure of this region. The oxygen-labile nature of the
TACYs is likely due to the reactive thiol group of the Cys
residue interacting with reactive groups on other proteins (35),
thus blocking participation of the undecapeptide region in the
cytolytic functions of the molecule. It is possible that confor-
mational changes in the variant PLO undecapeptide may pre-
vent occlusion of the undecapeptide by reactive proteins asso-
ciated with the Cys residue of His-PLO.C492. This hypothesis is
supported by the fact that modification of the free thiol group
of His-PLO.C492 with thiol blocking reagents results in a small
inhibitory effect but not the major effect seen with other
TACYs (21).

Trp residues within the undecapeptide of PFO are instrin-
sically involved in the interaction of this TACY with target cell
membranes (41) and may interact directly with cholesterol (32).
The changes in the PLO undecapeptide, particularly the inser-
tion of the Pro which alters the spacing between Trp
residues, are likely to change the conformation of the Trp
residues with respect to each other. The cholesterol inhibition
studies suggest that both native and recombinant PLOs and
LLO are inhibited by free cholesterol in a similar manner. Of
course, this does not necessarily indicate that the interactions
of PLO and other TACYs with the target membrane are simi-
lar.

Among the conserved residues over the entire amino acid

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**TABLE 2. Bacterial viable counts from passively immunized mice**

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Day</th>
<th>Blood</th>
<th>Liver</th>
<th>PF</th>
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<tr>
<td>1</td>
<td>4</td>
<td>&lt;1 × 10^7, &lt;1 × 10^3</td>
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<td>&lt;1 × 10^3, &lt;1 × 10^3</td>
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<tr>
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<td>13</td>
<td>&lt;1 × 10^7, &lt;1 × 10^3</td>
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<tr>
<td>2</td>
<td>4</td>
<td>1.0 × 10^7, &lt;1 × 10^3</td>
<td>4.2 × 10^3, &lt;1 × 10^3</td>
<td>3.6 × 10^3, 3.0 × 10^4</td>
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<td>6.0 × 10^7, 7.4 × 10^3</td>
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<td>4</td>
<td>&lt;1 × 10^2, 9.5 × 10^3</td>
<td>6.7 × 10^{10}, 5.3 × 10^7</td>
<td>1.0 × 10^9, 1.9 × 10^9</td>
</tr>
</tbody>
</table>

*a* Mice immunized with α-His-PLO (group 1), preimmune serum (group 2), or PBS (group 3) were challenged with 2.3 × 10^6 CFU of log-phase A. pyogenes BBR1. Bacterial viable counts were determined from the blood, liver, and PF of individual mice euthanized on days 4, 7, and 13 postchallenge or when moribund.

*b* Viable counts were measured as CFU per milliliter of blood, CFU per gram of liver, or CFU per milliliter of PF. The limits of detection were 10^2 CFU/ml for blood for PF and 10^3 CFU/g for liver.

*c* Two mice died, and bacterial counts were not performed.
sequences of members of the TACY family, there are high numbers of conserved Tyr (10 residues), Pro (8 residues), and Trp (6 residues) residues with respect to their frequencies of occurrence in TACYs. Many of the Trp residues are in the conserved undecapeptide or a region associated with complement activation by PLY (31). Interestingly, PLO and other TACYs have residues which are associated with this function in PLY (Fig. 3B) (8, 31), although only SLO has been demonstrated to bind complement (7). The conserved Pro residues are of considerable interest because of the propensity of this residue to form bonds in protein molecules (9). Pro residues are often located at the limits of functional domains and are intimately involved in protein folding and tertiary structure (9).

In addition to the eight totally conserved Pro residues, another five are conserved in at least 8 of the 10 TACY protein sequences. We propose that the conserved Pro residues are important in the conformation of TACYs, either in determining their monomeric forms or in exposing sites for oligomerization within target membranes. Only the C-terminal Pro has been mutagenized in PLY, but this resulted in a significant decrease in the ability of PLY to bind to erythrocyte membranes (34). Many TACYs are involved in the pathogenic mechanisms of their producers. Knockout mutations in genes encoding PLY and LLO result in significant attenuation of the virulence of S. pneumoniae (5) and L. monocytogenes (15) in mouse models. LLO has been particularly well studied and is required for the pneumoniae and LLO result in significant attenuation of the virulence of L. monocytogenes (15) in mouse models.

The precise role of PLO in the pathogenesis of A. pyogenes is still unknown. Construction of, and mouse challenge with, an isogenic plo mutant will allow more precise determination of the role of PLO in virulence. These experiments will be facilitated by the recent development of an electroporation protocol for A. pyogenes (22). Vaccination of mice and other target animals, such as cattle, with toxoided recombinant PLO preparations, followed by challenge, will allow determination of the efficacy of immunization with PLO for host protection. These experiments are currently being undertaken in this laboratory.

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