Cell Surface Protein of *Pseudomonas (Hydrogenomononas) facilis*

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Intact cells of *Pseudomonas facilis* contain one major molecular weight class of protein that is exposed at the cell surface as revealed by lactoperoxidase-catalyzed iodination with \(^{125}\)I. All molecular weight classes of protein in derived cell envelope preparations are apparently saturated by iodination by lactoperoxidase after prolonged sonic treatment. The molecular weight of the predominantly exposed protein in intact cells is approximately 16,000, which is the minimal molecular weight of a cell envelope protein that precipitates as a complex with phospholipid from extracts of *P. facilis*. The isolation of labeled phospholipoprotein (PLP) after labeling intact cells with \(^{125}\)I corroborates previous experiments which suggested a surface location for the protein portion of the phospholipoprotein (P\(_{PLP}\)). Solvent extraction of cells and immunological evidence, including studies with ferritin-coupled antibodies, indicate that P\(_{PLP}\) is located at the cell surface and may also be within the cell envelope. These experiments suggest that P\(_{PLP}\) is the major cell surface protein in *P. facilis*.

Although proteins are known to be present in considerable amounts in the cell envelope of gram-negative bacteria, little is known of these components (7, 9, 19). In particular, the protein in the outer membrane of gram-negative bacteria, which amounts to about 60% of the cell envelope protein in *Salmonella typhimurium* (20), has been investigated only recently. Polyacrylamide gel electrophoresis of purified outer membrane preparations from gram-negative bacteria has revealed the presence of up to 11 proteins including one or two major proteins which may account for as much as 70% of the total protein in this outermost cell structure (2, 20, 28). Selective proteolytic attack of the outer membrane of *Escherichia coli* has been used as evidence for an asymmetric arrangement of proteins in the membrane and for the presence of select molecular weight classes of proteins exposed at the membrane surface (2). The facile release of lipopolysaccharide (LPS)-protein-lipid complexes from some gram-negative bacteria (5, 10, 15) indicates the lability of surface components of the outer membrane and the presence of protein associated with lipid and LPS on the surfaces of these organisms.

Previous work (11, 14, 25) in this laboratory on a hydrophobic phospholipoprotein (PLP), which precipitates when the ionic strength of the "soluble" fraction from *Pseudomonas facilis* is lowered, revealed a cell envelope location for the protein component (P\(_{PLP}\)). P\(_{PLP}\) was unusually hydrophobic, apparently pure, and had a minimal molecular weight of 15,000 (25). Anti-PLP, which was directed towards P\(_{PLP}\), agglutinated *P. facilis*, establishing that the protein was located in part on the cell surface (25). In this paper we present immunological and chemical evidence that P\(_{PLP}\) is located in the outer membrane portion of the cell envelope of *P. facilis* and that a portion of it is very loosely bound on the cell surface. By using the enzyme lactoperoxidase, shown by Phillips and Morrison (21) to catalyze radioiodination of proteins exposed on the membrane surface, we present data which indicate that P\(_{PLP}\) is the major cell surface protein in *P. facilis*.

**MATERIALS AND METHODS**

**Organism and medium.** The origin, maintenance, and culture of *P. facilis*, previously classified as *Hydrogenomononas facilis* (23), were as described by Kuehn and McFadden (13). Cultures were grown in 300 ml of fructose medium, transferred to 4.5 liters of the same medium, grown at 30°C with slow shaking (160 rpm), and harvested at the late log phase of growth.

**Materials.** Lysozyme, pepsin, albumin (bovine, fraction V), carbonic anhydrase, lactoperoxidase, Tri-

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ton X-100, agarose, and Coomassie Brilliant Blue R were obtained from Sigma Chemical Co., St. Louis, Mo.; carrier-free $^{131}$iodine was from Schwarz/Mann Co., Orangeburg, N.Y.; ferritin (horse spleen, twice crystallized), tolune-2,4-diisocyanate, goat anti-rabbit immunoglobulin G (IgG) serum, and fluorescein iso-thiocyanate-conjugated goat anti-rabbit (IgG fraction) were from Miles Laboratories, Inc., Kankakee, Ill.; and sodium dodecyl sulfate (SDS; sequential grade) was from Pierce Chemical Co., Rockford, Ill. All other chemicals were reagent grade or the purest grade available.

Cell envelope, spheroplast, and spheroplast membrane preparations. Cell envelope preparations of *P. faciles* were obtained from cells in the late log phase essentially by the method of Schmitt (27). Wet-packed cells were brought to a 50% (wt/vol) suspension in 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-chloride buffer (pH 8.0, 25 C) and broken by passage through a French pressure cell at 2 C and 15,000 lb/in$^2$. The treated suspension was centrifuged at 2 C at 3,000 × g for 5 min to remove unbroken cells. The supernatant portion was then centrifuged at 100,000 × g for 60 min at 2 C, and the particulate fraction was collected and washed once with the same Tris buffer.

Ethylenediaminetetraacetate (EDTA)-lysozyme spheroplasts were prepared by the method of Birdsell and Cota-Robles (1). Spheroplast membranes were obtained by osmotic shock of the EDTA-lysozyme spheroplast preparation by a 10-fold dilution of the spheroplast suspensions into deionized water.

Iodination procedure. The general procedure of Poduslo et al. (22) was followed. Samples were suspended in 5.0 ml of 0.1 M sodium phosphate (pH 7.4), and lactoperoxidase was added to the reaction mixture to give a final concentration of 1 μM. Either 50 or 100 μCi of Na$^{131}$I (sp act 1 mCi/mmol) were added, and the reactions were initiated and continued at 30 C by the addition of 0.02 ml of 8 mM H$_2$O$_2$ at 15-s intervals. The addition of small amounts of H$_2$O$_2$ was done to minimize undesirable oxidation of intact bacteria or cell envelope preparations by free peroxidase. After either 60 or 90 min, the reactions were quenched by chilling to 2 C, and the samples were dialyzed overnight at 2 C against the phosphate buffer. Particles were recovered from particulate samples by centrifugation at 100,000 × g (2 C) for 10 min and stored with soluble samples at −20 C until application to SDS polyacrylamide gels and subsequent electrophoresis. The following samples were radioiodinated for 90 min (total addition of 7.2 ml of H$_2$O$_2$ solution): intact *P. faciles* which had been harvested at late log phase (100 μCi of $^{131}$I), derived cell envelope preparations sonicated treated for 15 min (100 μCi of $^{131}$I), and derived cell envelope preparations sonicated treated for 15 min (100 μCi of $^{131}$I). Other samples including intact *P. faciles* which had been harvested at late log phase (50 μCi of $^{131}$I), derived cell envelope preparations sonicated treated for 15 min (50 μCi of $^{131}$I), PLP, bovine serum albumin (BSA), and lysozyme were incubated in the presence of Na$^{131}$I for 60 min (total addition of 4.8 ml of H$_2$O$_2$ solution).

Gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out by the method described by Osborn et al. (30). The gels were removed, fixed in a 15% acetic acid-25% isopropanol solution for 12 h, and then placed in a 10% acetic acid-25% isopropanol solution for 6 h before staining for 12 h with a solution of 0.25% Coomassie Brilliant Blue containing 25% isopropanol and 10% acetic acid. The gels were destained by soaking in 10% acetic acid. Alternatively, 125-mm gels containing radioiodinated material were sliced laterally in 1.20-mm sections after electrophoresis, and the gamma emissions were counted for each gel section with a gamma-spectrometer. Molecular weights of the components in stained gels or radioactive gels were estimated by comparison with migration of standards under identical conditions with the assumption that standards and unknowns were saturated by 2% SDS and that resultant structures were all in an extended rod-like configuration (24).

Immunological procedures. The preparation of antibody to PLP, Ouchterlony double diffusion, immunoelectrophoresis, and agglutination of bacteria by antibody were as described previously (25). Antiserum to phenol-extracted protein or cell envelope preparations was obtained from rabbits after injecting approximately 4 mg subcutaneously twice weekly for 3 weeks and bleeding 1 week after the final injection. Ferritin-labeled antibody studies employed either (i) antibody obtained from fractionation of antiserum to PLP with ammonium sulfate (25) or (ii) goat anti-rabbit IgG serum conjugated to ferritin by using the bifunctional reagent, tolune-2,4-diisocyanate (29).

For best results it was found that conjugated antibody-ferritin should be prepared and used for electron microscope studies within 1 day. For the direct ferritin-coupled antibody studies, the following preparations derived from late log *P. faciles* were incubated with ferritin-labeled anti-PLP in 0.01 M Tris-chloride buffer (pH 8.0, 25 C) at 37 C for 4 h and then at 2 C for 24 h: lysozyme-EDTA spheroplasts from plasmolyzed cells, phenol-extracted intact cells, and spheroplast membranes derived from lysozyme-EDTA spheroplasts. The Tris buffer solution for the lysozyme-EDTA spheroplasts contained 0.5 M sucrose to prevent lysis of the spheroplasts. The above samples were centrifuged at 2,000 × g for 10 min at 2 C, washed in the same cold Tris buffer, and pelleted at 5,000 × g at 2 C. The pelleted samples were then prepared for electron microscope examination. Normal rabbit serum conjugated to ferritin was used for the control samples.

For the indirect ferritin-coupled antibody method, *P. faciles* cells were incubated with antibody to PLP in 0.01 M Tris-chloride (pH 8.0, 25 C) for 4 h at 37 C and then for 24 h at 2 C. The cells were washed twice with the same cold Tris buffer at 2 C and incubated with ferritin-coupled goat anti-rabbit IgG serum in the Tris-chloride buffer for 4 h at 37 C and 24 h at 2 C. After centrifugation at 2,000 × g for 10 min (2 C), the cells were washed twice with the cold Tris buffer, and the 5,000 × g pellet (2 C) was prepared for electron microscopy. In the control sample, *P. faciles* cells were incubated initially with normal rabbit serum instead of antibody to PLP.

The indirect method was used for the immunofluorescence study of *P. faciles*. Cells which had been
grown heterotrophically on fructose to early log phase
or late log phase or autotrophically with 90% H2, 5% O2,
and 5% CO2 by the procedure of Kuehn and Mc-
Fadden (13) were mixed with antibody to PLP in 0.01
M Tris-chloride (pH 8.0, 25 C) and incubated for 2 h
at 40 C. The cells were then washed twice with the Tris
buffer at 2 C and resuspended in the same buffer
containing fluorescein isothiocyanate-conjugated goat
anti-rabbit (IgG fraction). After incubation at 40 C
for 2 h, the stained cells were mounted on a microscope
slide by using a mounting agent composed of 9 vol of
glycerol per 1 vol of 0.05 M sodium phosphate
buffer (pH 7.3) containing 0.85% NaCl. For control samples,
normal rabbit serum was used instead of anti-PLP in
the first incubation step. The samples were examined
under oil immersion by fluorescence microscopy with a
Zeiss Fluorite 100 × objective. The light source was the
mercury vapor lamp and dark-field illumination was
used.

Electron microscopy. Samples were prepared for
the electron microscope as previously described (11).

Solvent extraction procedures. Various solvent
extractions were used to aid in locating PPLP within
the complex layers of the cell envelope and finding the
condition required to dissociate PPLP from cells enve-
lope preparations and intact cells.

Phenol extraction of intact P. facilis was conducted
by the general procedure of Weidel et al. (31). A 5-g
amount of P. facilis was suspended in 20 ml of
phenol-water (1:1, vol/vol) at 65 C for 10 min. The
mixture was then cooled in an ice bath and cen-
trifuged at 20,000 × g. After separation from the
aqueous phase, the phenol phase was dialyzed at 2 C
first against 50 vol of 0.01 M Tris-chloride (pH 8.0,
25 C) containing 1% Triton X-100 and 1 M urea and
finally against 0.01 M Tris-chloride (pH 8.0, 25 C)
to remove most of the residual phenol. The resultant
precipitate is referred to as phenol-extracted protein.
Residual phenol was also removed from phenol-ex-
tracted P. facilis cells by the above dialysis procedure.

P. facilis cells were also extracted by either (i)
suspension of 1 g of late log-phase cells in 1 ml of 0.9% saline
at 25 C for 5 min and collection of the supernatant
fraction from centrifugation at 100,000 × g at 2 C for 10 min or
(ii) incubation of 1 g of late log

RESULTS

Iodination experiments. When intact P. fa-
cilis is labeled with 125I and derived cell
envelopes are examined by gel electrophoresis, a
select size of protein (mol wt 16,000) is predomi-
nantly labeled (Fig. 1). This experiment was
performed with both 100 μCi and 50 μCi of
125I iodide. The similar gel profile for both experi-
mints indicates lactoperoxidase-catalyzed equilibration
of 125I with all proteins exposed to the
surface of intact cells.

The gel profile of cell envelope protein labeled
after isolation of the cell envelope fraction from
intact cells (Fig. 2) also reveals select molecular
weight classes of protein exposed to the enzyme
lactoperoxidase. The incorporation of relatively
little label into this preparation is similar to that
in experiments with intact cells, as is the
predominant labeling of protein of a molecular
weight of about 16,000. More proteins, however,
are labeled in this fraction than in intact cells.
Presumably this results from iodination of pro-
teins at the cytoplasmic face of the cell enve-
lope.

Extensive incorporation of 125I into protein was
found (Fig. 3) when the cell envelope fraction was
sonically treated prior to reaction with radioidine and lactoperoxidase. A brief
sonic treatment (30 s) apparently exposes more
of the cell envelope protein since considerable
iodine is incorporated into all molecular weight
classes of protein, although a major peak corre-
sponding to a molecular weight of about 16,000
is still evident. More extensive sonic treatment
(15 min) exposes still more protein as evidenced
by the higher incorporation of label in more cell
envelope molecular weight classes (Fig. 3). The
very similar gel profile of cell envelope protein
labeled with either 50 μCi or 100 μCi of 125I after
the 15-min sonic treatment indicates equilibra-
tion of label with exposed proteins. As a con-
sequence of longer sonic treatment, protein of
16,000 daltons becomes a relatively minor radio-
active peak in marked contrast to the experi-
ments with intact cells.

Cell and cell envelope samples incubated with
100 μCi of Na125I in the absence of lac-
toperoxidase incorporated very little label
(1–2% of lactoperoxidase-treated samples). The
gel profiles (not shown) of these samples
revealed only one small peak which was located
slightly ahead of the tracking dye. This may
represent non-enzymatic iodination of lipid.

When intact P. facilis was labeled with 100
μCi of 125I by using lactoperoxidase, the PLP
subsequently isolated by the method of Kuehn
et al. (14) contained gamma emissions of 2,050
counts per min per mg of protein (16). Extrac-
tion of lipid from the labeled PLP preparation as
described by Kuehn et al. (14) yielded material
having 1,565 counts per min per mg of protein,
demonstrating that the protein component of
PLP in intact P. facilis contained most, if not
of BSA, aldolase, pepsin, carbonic anhydrase, and lysozyme in gels stained with Coomassie Brilliant Blue.

Ferritin-coupled antibody and immunofluorescence studies. Fig. 5A shows the specific labeling of the cell surface of intact P. facilis (incubated initially with antibody to PLP) with ferritin-coupled goat anti-rabbit IgG serum. The control sample (Fig. 5B), which had been incubated initially with normal rabbit serum, contained small amounts of ferritin with no localized concentration on the cell surface.

The direct method, using ferritin-coupled antibody to PLP, resulted (not shown) in a small amount of label specifically attached to the cell surface with moderate amounts of scattered ferritin, whereas the control sample contained essentially no ferritin label. This indicated that the antigenic determinants of PPLP on the cell surface were sterically less available to the very large ferritin-coupled anti-PLP molecules used in the direct method than to the relatively smaller unconjugated anti-PLP used in the initial step of the indirect method. Alternatively, the antibody-antigen complex may be more easily detached from the cell surface when the direct method is employed; this would explain the presence of scattered ferritin in the samples in which ferritin-labeled antibody to PLP had been used.

In contrast, phenol-water-extracted P. facilis contained practically no ferritin attached to the
FIG. 3. Iodination of sonically treated cell envelope fractions each derived from 1 g of wet-packed *P. facilis*. The cell envelope fractions were sonically treated for the indicated time intervals at 20 kc before reaction with $^{125}$I. The $^{125}$I distribution was determined after SDS-polyacrylamide gel electrophoresis. Symbols: cell envelope fraction sonically treated for 30 s and labeled by using 50 μCi of $^{125}$I, ○; cell envelope fraction sonically treated for 15 min and labeled by using 50 μCi of $^{125}$I, Δ; cell envelope fraction sonically treated for 15 min and labeled by using 100 μCi of $^{125}$I, O. For other specifications see Fig. 1.

FIG. 4. Iodination of PLP, BSA, and lysozyme. PLP, BSA, and lysozyme were labeled by using 100 μCi of $^{125}$iodide, and the distribution of radioactivity was determined after SDS-polyacrylamide gel electrophoresis. Symbols: PLP, ○; BSA, Δ; lysozyme, O. For other specifications see Fig. 1.
FIG. 5. Electron micrographs of *P. facilis* with ferritin-coupled goat anti-rabbit IgG serum. A, Cells were preincubated with antibody to PLP; no post-stain. B, Cells were preincubated with control serum. Bar markers in this and all electron micrographs represent 0.5 \( \mu \)m.
extracted surface (not shown). Large masses of scattered ferritin were visible, however. Since PPLP can be partially eluted from the intact cells with various solvent techniques, including phenol-water extraction, the scattered ferritin may have been due to attachment to fragments of the cell wall containing PPLP. The presence of almost no scattered ferritin in the control sample was consistent with this possibility.

Spheroplast membranes prepared from EDTA-lysozyme spheroplasts (1) by osmotic shock show very heavy label with ferritin-coupled antibody to PLP (Fig. 6A), whereas the control sample (Fig. 6B) contains practically no ferritin. The fragmentation of the EDTA-spheroplasts exposes considerably more antigenic determinants of PPLP compared with spheroplasts (not shown) and affords heavy labeling by the bulky ferritin-coupled anti-PLP molecules.

Late log-phase P. facilis cells were labeled indirectly with fluorescein-conjugated goat anti-rabbit IgG (not shown) in a uniform manner. This again indicates the presence of uniformly distributed PPLP on the cell surface. In the control sample (preincubation with normal rabbit serum) the cells were not stained. P. facilis which had been grown heterotrophically on fructose to early log phase or autotrophically with H2, O2, and CO2 were also stained with fluorescein-conjugated goat anti-rabbit IgG.

**Solvent extraction experiments.** Immunoelectrophoresis with anti-PLP and the protein extracted from intact P. facilis with phenol resulted in a single, long precipitin arc located slightly toward the cathode (Fig. 7A). More significantly, Fig. 7B shows the fusion of precipitin lines resulting from double diffusion of phenol-extracted protein and PLP against antibody to PLP. The aqueous layer was found to contain large amounts of saccharide measured as hexose (8), presumably as LPS, and small amounts of protein (16) in a ratio of 9:1 by weight, in accord with the data of Weidel et al. (31) for E. coli. Antiserum prepared against phenol-extracted protein also yielded a precipitin line of identity between PLP and phenol-extracted protein after double diffusion (not shown).

Both P-1 and P-2 fractions from chloroethanol extraction of cell envelopes yielded precipitin lines of identity with PLP after double diffusion against antibody to PLP (Fig. 7B). The precipitin reaction with P-1 indicates that some PPLP is associated tightly with the cell envelope structure since P-1 represents material which can not be extracted with chloroethanol-water. Interestingly, hexose (0.20 mg per mg of protein), pentose (0.22 mg per mg of protein), and phosphate (0.02 mg per mg of protein) were found in the P-1 fraction, indicating the possibility of covalent attachment of carbohydrate and lipid to protein.

The presence of some PPLP which is very tightly bound to intact cells is indicated by the agglutination of heat-treated P. facilis (Table 1). Considerable amounts of this protein may be denatured or extracted, or both, by the heat treatment, however, since the titer is reduced from 2,048 for untreated cells to 256 for heat-treated cells. Antiserum either to phenol-extracted protein or cell envelopes agglutinates intact P. facilis (Table 1). P. facilis extracted by the phenol-water procedure is not agglutinated by antibody to PLP, indicating that the removal of the outer membrane removes all of the exposed antigenic sites.

A portion of the PPLP on the cell surface is probably very loosely bound as indicated by the mild 0.9% saline extraction of intact cells at 25 C which removes material yielding a precipitin line of identity with PLP after double diffusion against anti-PLP (Fig. 7B, well no. 5).

**Cell envelope protein composition.** The protein composition of cell envelope preparations from P. facilis (Fig. 3 and 8) is quite similar to the cell envelope protein compositions reported for E. coli (2, 27) and Salmonella typhimurium (20). A very heterogeneous pattern of proteins is obtained with major protein peaks located at approximate molecular weights of 34,000 and 42,000. Protein of a molecular weight of 16,000 is about 8% of the total cell envelope protein on the basis of 125I incorporation into cell envelope preparations that had been sonically treated for 15 min (Fig. 3). This calculation assumes equal fractions of tyrosine and histidine residues in all the cell envelope proteins since the enzyme lactoperoxidase specifically iodinates only these amino acid residues (21). From a scan of a Coomassie Brilliant Blue-stained gel, protein of this size (arrow, Fig. 8) was estimated to compose about 12% of the total cell envelope protein. This calculation is also approximate because the absorbance of Coomassie Brilliant Blue-stained protein deviates from Beer’s law at higher protein concentrations (3). Both these calculations indicate, however, that protein of a molecular weight of 16,000 is present in P. facilis cell envelopes in substantial amounts although it is not the major protein species.

**DISCUSSION**

The radioiodination studies of intact cells, envelopes, and sonically treated envelopes of P. facilis provide evidence for a very asymmetric distribution of protein at the cell surface. In
Fig. 6. Electron micrographs (no post-stain) of spheroplast membranes derived from EDTA-lysozyme spheroplasts of P. facilib. A, Ferritin-coupled antibody to PLP was used; B, ferritin-coupled gamma-globulin from control serum was used.
The buffer (pH 8.0) containing 1% Triton X-100 was reacted with antibody to PLP in center trough. Electrophoresis was conducted for 30 min in 0.05 M Tris-chloride buffer (pH 8.0) at 5 mA per side. Ouchterlony double-diffusion experiment of materials extracted from intact cells and cell envelopes of *P. facilis*. The center well contained antibody to PLP, and the sample wells contained the following: (1) PLP, (2) phenol-extracted protein from *P. facilis*; (3) chloroethanol-insoluble material (P-1); (4) precipitate (P-2) obtained during dialysis of chloroethanol extracts of cell envelopes; (5) saline extract of intact *P. facilis*; and (6) aqueous layer from phenol-water extraction of intact *P. facilis*.

![Phenol extract](image)

**Fig. 7.** Immunoelectrophoresis and immunodiffusion experiments of extracted materials from *P. facilis*. The 1.5% agarose slides were made with 0.05 M Tris-chloride buffer (pH 8.0) containing 1% Triton X-100 and 1 M urea. A, Immunoelectrophoresis of phenol-extracted protein from *P. facilis* (left and right wells) reacted with antibody to PLP in center trough. Electrophoresis was conducted for 30 min in 0.05 M Tris-chloride buffer (pH 8.0) at 5 mA per side. B, Ouchterlony double-diffusion experiment of materials extracted from intact cells and cell envelopes of *P. facilis*. The center well contained antibody to PLP, and the sample wells contained the following: (1) PLP, (2) phenol-extracted protein from *P. facilis*; (3) chloroethanol-insoluble material (P-1); (4) precipitate (P-2) obtained during dialysis of chloroethanol extracts of cell envelopes; (5) saline extract of intact *P. facilis*; and (6) aqueous layer from phenol-water extraction of intact *P. facilis*.

Table 1. Agglutination of *P. facilis*

<table>
<thead>
<tr>
<th>Pretreatment of <em>P. facilis</em></th>
<th>Antibody directed against</th>
<th>Titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>PLP</td>
<td>2,048</td>
</tr>
<tr>
<td>At 100°C for 15 min in 0.1 M Tris-chloride buffer (pH 8.0) containing 0.005 M EDTA</td>
<td>PLP</td>
<td>256</td>
</tr>
<tr>
<td>Phenol-water-extracted cells</td>
<td>Phenol-extracted protein fraction</td>
<td>No agglutination 256</td>
</tr>
<tr>
<td>None</td>
<td>Cell envelope fraction</td>
<td>4,096</td>
</tr>
</tbody>
</table>

*In all cases, *P. facilis* was harvested at the late log phase of growth for use in the agglutination studies.  
*Titer is the reciprocal of the highest dilution of antibody that still gives an observable agglutination.*

The incorporation of $^{125}$I into P$_{PLP}$ upon labeling intact *P. facilis* is further evidence of a cell surface location for this protein. The relative mobility of $^{125}$I-labeled PLP in 2% SDS polyacrylamide gels after electrophoresis corresponds to the major radioactive peak (mol wt 16,000) obtained with cell envelopes from labeled intact cells. The presence of more than one protein species in the peak, of course, remains a possibility. The dense ferritin labeling of intact cells with ferritin-coupled goat anti-rabbit IgG serum (indirect method) suggests the presence of high levels of the protein at the cell surface. The small amount of attached ferritin on intact cells with the direct method...
(ferritin-coupled anti-PLP) may be a consequence of a high degree of steric hindrance to the approach of very bulky molecules to \( P_{PLP} \). In this situation, the initial binding of anti-PLP (indirect method) may then be favored over the direct binding of the more bulky ferritin-coupled anti-PLP molecules. The specific heavy labeling of spheroplast membranes directly with ferritin-coupled anti-PLP is consistent with this interpretation since the fragmentation of spheroplasts would be expected to expose more protein. The heavy ferritin labeling of spheroplast membranes also indicates that \( P_{PLP} \) is present in substantial amounts in the cell envelope of \( P. \) facilis. It should be noted that double diffusion of spheroplast membranes and PLP against antibody to PLP previously revealed a single precipitin line of identity (25).

Phenol-extracted cells showed a lack of agglutination with anti-PLP and absence of attached ferritin-coupled anti-PLP, indicating the removal of the exposed protein from intact cells by phenol-water extraction. Apparently, phenol-water extraction of intact cells removes the outer membrane of \( P. \) facilis as is the case for Veillonella sp. (181). The presence of \( P_{PLP} \) in the phenol phase after phenol-water extraction of intact cells revealed by immunoelectrophoresis and immunodiffusion also indicates an outer membrane location for this protein. The aqueous phase of the phenol-water extraction consists mostly of saccharide (probably lipopolysaccharide) but does not contain this protein, reflecting the known extremely hydrophobic nature of the protein (25).

Partial dissociation of \( P_{PLP} \) from intact cells and cell envelope preparations by extraction methods (ranging from very mild to rather harsh) provides evidence for great variation in the degree of integration of this protein within the cell envelope. The presence of the protein in the insoluble fraction of chloroethanol-extracted cell envelopes (P-1) demonstrates that some of this protein is extremely tightly bound. In addition, the agglutination of cells of \( P. \) facilis after extraction at 100 C for 15 min in the presence of 5 mM EDTA demonstrates that some \( P_{PLP} \) cannot be removed from intact cells even with this harsh treatment and therefore must be quite strongly bound to, or integrated into, the cell envelope. On the other hand, some \( P_{PLP} \) can be eluted from intact cells by extraction with 0.9% saline at 25 C.

It is likely that, during cell growth and cell division, the components of the cell wall are in a dynamic state. At various stages of cell elongation and subsequent cell division, some cell wall components may be initially integrated within the cell wall but become increasingly labile as cell wall synthesis continues. This would result in release of surface components from the cell during cell growth. LPS which is located in the outer membrane and in part on the cell surface has been found associated with protein and lipid as a LPS-protein-lipid complex released from bacterial cells (4, 5, 10, 12, 26, 30, 32). Of special relevance is the finding by Weckesser et al. (30) that extraction of Rhodopseudomonas capsulata with 0.9% NaCl at 37 C for 2 h results in elution of a LPS-protein-lipid complex. Saccharide is also present in the mild saline extract of \( P. \) facilis which contains \( P_{PLP} \). The LPS-protein-lipid complexes found in other laboratories may represent a structure which exists in the outer membrane in vivo. It will be of interest to see whether \( P_{PLP} \) is associated with LPS as a LPS-protein-lipid complex after mild elution from intact \( P. \) facilis. Whether related proteins occur in the complete somatic O antigens of various gram-negative bacteria will also be of deep interest since it has long been known that protein is a component of O antigens (17). The widespread occurrence of proteins similar to that of \( P_{PLP} \) on bacterial cell surfaces has been inferred recently on the basis that anti-PLP agglutinates a wide variety of bacteria (H. G. Rittenhouse, J. B. Rodda, and B. A. McFadden, J. Bacteriol., in press).

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**LITERATURE CITED**


