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# Cell Walls of *Pseudomonas* Species Sensitive to Ethylenediaminetetraacetic Acid

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Cell walls of 12 pseudomonads considered to be sensitive to ethylenediaminetetraacetic acid (EDTA) were prepared and analyzed. The wall of each species contained protein, peptidoglycan, loosely bound lipid, and lipopolysaccharide. The walls of *Pseudomonas stutzeri* and *P. syncyanea* were unusually susceptible to mechanical disintegration. The wall of *P. syncyanea* had an unusually high content of lipid and low contents of protein and peptidoglycan. Except for *P. syncyanea*, all the walls contained less phosphorus than the walls of the highly EDTA-sensitive *P. aeruginosa* and *P. alcaligenes*, but more than the walls of EDTA-resistant pseudomonads. The amino acid compositions of wall proteins were similar for all species. Amino sugars detected were glucosamine, galactosamine, muramic acid, and at least five unidentified components (possibly including fucosamine and quinovosamine). Glucose and rhamnose were the major neutral sugars in most walls. Galactose, mannose, fucose, and ribose were also detected, the last two each in a single species. Except for *P. stutzeri* and *P. syncyanea*, the walls had rather low contents of phospholipids (mainly cardiolipin, phosphatidylethanolamine, and phosphatidylglycerol in all species). An ornithine-containing nonphospholipid was present in all walls, and a hexuronosyldiglyceride was probably present in most walls. The fatty acid compositions of loosely bound lipids were qualitatively similar for all species: saturated $C_{16}$ and monoenoic $C_{16}$ and $C_{18}$ acids were the major components. Except for *P. aureofaciens*, the extraction of phosphorus on treatment of walls with EDTA at *p*H 9.2 was much less than for *P. aeruginosa* and *P. alcaligenes*.

Although the cell walls of gram-negative bacteria have been studied extensively in recent years, little attention has been paid to pseudomonads other than *Pseudomonas aeruginosa*. One reason for interest in this species lies in its exceptional sensitivity to ethylenediaminetetraacetic acid (EDTA). The toxicity of EDTA for *P. aeruginosa* (13, 20, 35, 51) and also for *P. alcaligenes* (20, 51) is a result of the extraction of essential components from the walls of the organisms (10, 21, 32, 42). For *P. aeruginosa*, the extract is a complex of lipopolysaccharide, protein, and loosely bound lipid (10, 21, 42; N. A. Roberts, G. W. Gray, and S. G. Wilkinson, *unpublished data*). In an attempt to elucidate the chemical basis for sensitivity of organisms to EDTA, the walls of *P. aeruginosa* (7-9, 14, 21), *P. alcaligenes* (21, 32), and pseudomonads resistant to EDTA (53) have been studied in these laboratories. This paper describes the results of a broad, comparative study of the walls of 12 other EDTA-sensitive organisms (51).

## MATERIALS AND METHODS

**Organisms and preparation of cell walls.** Cell walls were prepared from *P. aureofaciens* (NCIB 9030), *P. chlororaphis* (NCTC 7357), *P. denitrificans* (NCIB 8376), *P. fluorescens* (NCTC 10038), *P. fragi* (NCIB 8542), *P. mucidolens* (NCTC 8068), *P. ovalis* (NCTC 912), *P. putida* (NCIB 9034), *P. stutzeri* (NCIB 9040), *P. syncyanea* (NCTC 9943), *P. synxantha* (NCIB 8178), and *P. taetrolens* (NCIB 9396). Organisms were grown under the same conditions as those tested for sensitivity to EDTA (51). The washed organisms were disintegrated and the cell walls were purified by the methods described previously (21, 53), except that for single batches of *P. stutzeri* and *P. syncyanea* disintegration using an MSK homogenizer (B. Braun, Melsungen, Germany) was carried out for 1 min instead of the usual 3 min. For reference purposes, walls were also prepared from *P. aeruginosa* (NCTC 1999) grown both on Nutrient Agar (Oxoid) and on Tryptone Glucose Extract Agar (Oxoid) for 24 hr at 37 C.

**Analytical methods.** Most of the methods used were described previously (53). Additional paper chromatography for the identification of 2,6-diamino-

pimelic acid was done by using methanol-water-10 N HCl-pyridine [32:7:1:4, v/v (40)]. Butan-1-ol-benzene-formic acid-water [100:19:10:25, v/v, upper phase (17)] was used as an additional solvent for the identification of neutral sugars. Ninhydrin degradation of amino sugars was done by the method of Spiro (48). Methyl esters of fatty acids (free and ester-bound) present in readily extractable lipids were prepared by treatment with $BF_3$-methanol for 10 min at 100 C (36), and were analyzed by gas-liquid chromatography using polar and nonpolar columns (52). Extractions of cell walls with chloroform-methanol (2:1, v/v) and with EDTA at *p*H 9.2 were done as described previously (53). Lipids were also extracted from Nutrient Agar (Oxoid) by stirring with chloroform-methanol (2:1, v/v) for 2 hr at room temperature; the crude lipid extract was purified by washing with water (16).

## RESULTS

**General observations.** The walls of *P. syncyanea* and *P. stutzeri* differed from those of the other organisms both in the unusual difficulty with which they were deposited from suspension by centrifugation, and in their appearance in electron micrographs. To minimize loss of material into supernatant fluids, final washings of some batches of walls were done by centrifugation at 60,000 × *g* for 1 to 1.5 hr. In electron micrographs, the products isolated after disintegration of organisms for 3 min were apparently amorphous; no shapes corresponding to the envelopes of rod-shaped bacteria were detected. On decreasing the time of disintegration to 1 min, a greater proportion of intact cells remained, but the walls were still difficult to deposit. However, electron micrographs showed that the product from *P. syncyanea* consisted mainly of envelopes, often folded or wrinkled, and varying considerably in size (length about 1 to 9 μm). The corresponding product from *P. stutzeri* contained a much smaller proportion of relatively intact envelopes, and consisted mainly of smaller, circular particles (diameter about 0.1 to 0.2 μm). Walls from the remaining organisms were normal for gram-negative bacteria both in ease of deposition from suspension and in appearance in electron micrographs; all these products were adequately free from cytoplasmic contamination. The walls of *P. stutzeri* were yellow; other walls were off-white or cream-colored.

The infrared spectra of all species of wall were broadly similar to each other and to those of other gram-negative bacterial walls (21), although the spectrum for *P. syncyanea* contained an unusually strong band at 1,726/cm (associated with ester carbonyl absorption). Each of the spectra contained minor absorption bands in the range 900 to 940/cm, but these were generally less marked than the bands at 928/cm found for *P. aeruginosa* and *P. alcaligenes* (21).

**General analyses.** The results of quantitative analyses for nitrogen, phosphorus, and carbohydrate are given in Table 1. In this and succeeding tables, analyses relate to single batches of walls, and those for *P. syncyanea* and *P. stutzeri* relate to walls prepared by using 3-min disintegration, unless stated otherwise.

**Amino acid composition.** The walls of each species contained a complete range of amino acids expected for proteins, which are characteristically present in the walls of gram-negative bacteria. In general, the proportions of individual amino acids were similar for each organism. On a molar basis, alanine, aspartic acid, glutamic acid, and

TABLE 1. *General analyses of cell walls*[a]

| Species | Nitrogen | Phosphorus | Carbohydrate[b] | 2-Keto-3-deoxyoctonic acid | Aldoheptose[c] |
|---|---|---|---|---|---|
| *Pseudomonas aureofaciens* | 6.8 | 1.28 | 12.0 | 1.1 | 2.0 |
| *P. chlororaphis* | 8.3 | 1.42 | 9.1 | 1.3 | 1.2 |
| *P. denitrificans* | 7.4 | 1.65 | 10.0 | 1.7 | 1.1 |
| *P. fluorescens* | 6.8 | 1.42 | 15.1 | 1.2 | 0.7 |
| *P. fragi* | 8.2 | 1.70 | 6.4 | 1.1 | 1.2 |
| *P. mucidolens* | 8.4 | 1.63 | 6.0 | 1.1 | 1.7 |
| *P. ovalis* | 7.8 | 1.77 | 10.4 | 0.9 | 1.2 |
| *P. putida* | 7.4 | 1.52 | 6.2 | 1.1 | 1.1 |
| *P. stutzeri*[d] | 8.1 | 1.72 (1.59) | 5.0 (2.5) | 1.0 | 0.1 |
| *P. syncyanea*[d] | 5.4 | 2.43 (2.23) | 17.7 (12.5) | 1.1 | 1.2 |
| *P. synxantha* | 7.1 | 1.41 | 13.0 | 0.9 | 0.6 |
| *P. taetrolens* | 7.3 | 1.91 | 9.7 | 1.4 | 1.8 |

[a] Results expressed as percentage (dry weight).
[b] Determined by the phenol-$H_2SO_4$ method and expressed as glucose.
[c] Calculated as L-glycero-D-mannoheptose.
[d] Results in parentheses are for batches of walls prepared by using 1-min disintegration of cells.

glycine were usually the major components. In addition to amino acids derived from protein, 2,6-diaminopimelic acid (from peptidoglycan) and ornithine were present in all wall samples. The spread of analytical results obtained is given in Table 2. Including ammonia, amino sugars, ethanolamine, and trace components (cystine, methionine sulfoxide, and phosphates of ethanolamine or glucosamine, or both), the recoveries of wall nitrogen in amino components of acid hydrolysates were in the range 87 to 106%.

**Amino sugar composition.** The peptidoglycan components muramic acid and glucosamine were present in all wall samples. For most species, there was a substantial molar excess of glucosamine (also a component of lipopolysaccharide) over muramic acid. Galactosamine was probably present in all walls except those of *P. synxantha* and *P. mucidolens*. When the Technicon AutoAnalyzer was used, we found small peaks (corresponding to unknown II in Table 3) in about the position of galactosamine for *P. chlororaphis* and *P. aureofaciens*; because of small variations between chromatograms, an exact correspondence could not be confirmed. However, the formation of lyxose on ninhydrin degradation of hydrolysates from these and other walls believed to contain galactosamine indicated that unknown II was probably this amino sugar.

In addition to the three amino sugars described above, a series of unidentified, ninhydrin-positive components (unknowns I, III, IV, V, and VI), also suspected of being amino sugars, was detected. Like amino sugars, these components gave rather broad peaks on chromatograms from the AutoAnalyzer. Again like amino sugars, the positions of the peaks on chromatograms relative to those of neutral amino acids could be varied rather widely by small adjustments to the *p*H value of one of the buffers (nominally *p*H 2.875) used in making the elution gradient. Unknown I, present only in *P. aureofaciens*, was eluted between glucosamine and galactosamine, and unknown III, present only in *P. chlororaphis*, was eluted marginally later than unknown II (galactosamine?) in the same hydrolysate. By using a normal set of buffers (with which peaks for valine and galactosamine are superimposed), unknown IV was eluted in about the position of methionine, unknown V about 25 min later than unknown IV, and unknown VI about 25 min before isoleucine. Unknown IV (from *P. putida*) and unknown VI (from *P. fragi*) have been isolated, and the results of preliminary studies (to be reported separately) indicate that these compounds are, respectively, quinovosamine and fucosamine. For the purpose of calculating contents of amino sugars (Table 3), the color

TABLE 2. *Amino acid composition of cell walls*

| Amino acid | Range[a] |
|---|---|
| Aspartic acid | 2.24–4.85 |
| Threonine | 1.13–2.60 |
| Serine | 1.69–2.86 |
| Glutamic acid | 2.54–4.78 |
| Proline | 0.82–1.18 |
| Glycine | 1.03–2.24 |
| Alanine | 2.56–4.21 |
| Valine | 1.16–2.50 |
| Methionine | 0.33–0.90 |
| Isoleucine | 0.73–1.32 |
| Leucine | 2.17–3.08 |
| Tyrosine | 1.04–2.41 |
| Phenylalanine | 1.21–2.57 |
| Lysine | 1.20–2.20 |
| Histidine | 0.48–0.79 |
| Arginine | 1.45–2.37 |
| Ornithine | 0.44–1.56 |
| 2,6-Diaminopimelic acid | 0.53–2.21 |

[a] Results, expressed as percentage (dry weight), were calculated for residues of amino acids and were not corrected for destruction or slow release. Ranges are results for the 12 species studied.

yield with ninhydrin and the molecular weight used for unknowns I to VI were those of glucosamine. On this basis, unknown IV could be the major ninhydrin-positive component of the wall of *P. putida*.

**Sugar composition.** The neutral sugar components of walls which were identified by paper chromatography are given in Table 4, which also gives semiquantitative assessments of the relative amounts of different monosaccharides in each species of wall. The impressions of carbohydrate contents of walls obtained by inspection of chromatograms were in general agreement with the results of analyses for total carbohydrate obtained by the phenol-$H_2SO_4$ method (Table 1). Glucose was a component of all walls, although the amount for *P. stutzeri* was very small. Galactose also was probably present in most or all walls, but a conclusive identification was not possible in most cases because of the rather small amounts of the component and possible confusion with aldoheptoses (11). Rhamnose occurred in substantial amounts in most walls, but was absent from the walls of *P. denitrificans*, *P. taetrolens*, and *P. putida*. Mannose was present in the walls of *P. aureofaciens* and *P. denitrificans*, whereas fucose and ribose each occurred in a single species of wall (*P. denitrificans* and *P. syncyanea*, respectively). Although intensive centrifugation was used to deposit the walls of *P. syncyanea*, contamination by ribonucleic acid is unlikely to account for the presence of ribose. Thus, the walls were treated with pancreatic

TABLE 3. *Amino sugar and ethanolamine contents of cell walls*[a]

| Species | Glucosamine | Galactosamine | Muramic acid | Unknown I | Unknown II | Unknown III | Unknown IV | Unknown V | Unknown VI | Ethanolamine |
|---|---|---|---|---|---|---|---|---|---|---|
| *Pseudomonas aureofaciens* | 5.58 | 0(?) | 1.24 | 1.10 | 0.45 | 0 | 0 | 0 | 0 | 0.05 |
| *P. chlororaphis* | 2.56 | 0(?) | 1.51 | 0 | 0.50 | 0.21 | 0 | 0 | 0 | 0.10 |
| *P. denitrificans* | 1.95 | 1.25 | 1.27 | 0 | 0 | 0 | 0 | 0 | 1.29 | 0.14 |
| *P. fluorescens* | 2.13 | 0.50 | 0.71 | 0 | 0 | 0 | 0.50 | 1.07 | 0 | 0.31 |
| *P. fragi* | 2.14 | 0.45 | 0.83 | 0 | 0 | 0 | 0 | 0 | 1.59 | 0.31 |
| *P. mucidolens* | 2.98 | 0 | 1.17 | 0 | 0 | 0 | 1.09 | 0 | 0 | 0.32 |
| *P. ovalis* | 1.75 | 2.39 | 1.38 | 0 | 0 | 0 | 0 | 0 | 0 | 0.54 |
| *P. putida* | 3.12 | 0.59 | 1.47 | 0 | 0 | 0 | 7.35 | 0 | 0 | 0.30 |
| *P. stutzeri* | 2.22 | 0.72 | 0.72 | 0 | 0 | 0 | 0.45 | 0 | 0 | 0.79 |
| *P. syncyanea* | 1.74 | 0.53 | 0.25 | 0 | 0 | 0 | Trace | 0 | 0 | 1.25 |
| *P. synxantha* | 2.39 | 0 | 1.17 | 0 | 0 | 0 | 1.67 | 0.81 | 0 | 0.08 |
| *P. taetrolens* | 4.07 | 2.29 | 0.94 | 0 | 0 | 0 | 0 | 0 | 0 | 0.39 |

[a] Results, expressed as percentage (dry weight), were calculated for residues of amino compounds and were not corrected for destruction.

TABLE 4. *Neutral sugar components of cell walls*[a]

| Species | Galactose | Glucose | Mannose | Fucose | Ribose | Rhamnose |
|---|---|---|---|---|---|---|
| *Pseudomonas aureofaciens* | ++++ | ++++ | + | 0 | 0 | ++ |
| *P. chlororaphis* | + | +++ | Trace? | 0 | 0 | ++ |
| *P. denitrificans* | + | ++++ | ++ | ++++ | 0 | 0 |
| *P. fluorescens* | Trace? | + | 0 | 0 | 0 | +++ |
| *P. fragi* | + | ++++ | 0 | 0 | 0 | ++ |
| *P. mucidolens* | + | +++ | 0 | 0 | 0 | +++ |
| *P. ovalis* | + | +++ | 0 | 0 | 0 | ++++ |
| *P. putida* | + | ++++ | 0 | 0 | 0 | 0 |
| *P. stutzeri* | + | Trace | 0 | 0 | 0 | ++ |
| *P. syncyanea* | + | ++ | 0 | 0 | + | ++++ |
| *P. synxantha* | + | ++++ | 0 | 0 | 0 | ++++ |
| *P. taetrolens* | Trace? | ++++ | 0 | 0 | 0 | 0 |

[a] Amounts of components, relative to other components of the same cell wall, were judged by the size and intensity of spots obtained on paper chromatograms.

ribonuclease during purification, and in aqueous suspension (0.1 mg/ml) did not give an absorption peak or inflection at 260 nm. Also, ribose was absent from walls of *P. stutzeri* obtained by using the same conditions of centrifugation.

In addition to the sugars listed in Table 4, a trace of an unidentified, neutral reducing compound was found in hydrolysates from *P. denitrificans.* It had *R(Rhamnose)* 1.34 on paper chromatography using butan-1-ol–ethanol–water–ammonia (specific gravity 0.88; 40:10:49:1, v/v, upper phase). No attempt was made to identify aldoheptoses chromatographically, but the results of colorimetric analyses for these compounds (Table 1) indicated that they were present in all walls. Positive results were also obtained in analyses for 2-keto-3-deoxyoctonic acids for each of the walls (Table 1). In these analyses, the spectrum of the reaction products (thiobarbituric acid test) contained the expected absorption maximum at about 548 nm in all cases except *P. denitrificans,* for which it was at about 538 nm. The presence of other sugar acids in most walls was indicated by studies on their loosely bound lipids.

**Lipid composition.** The contents of loosely bound lipids, extracted from walls using chloroform-methanol (2:1, v/v) at room temperature, are recorded in Table 5. In general, the values are fairly high, even for gram-negative bacteria (45), and the lipid content of the walls of *P. syncyanea* is exceptionally high. Nonlipid contaminants (probably only protein) were present in all extracts, as shown by the failure of some solids to redissolve in chloroform-methanol after drying the extracts. A complete range of protein amino acids, in small amounts, was found in each lipid sample, and quantitative analyses showed that these components accounted for nearly 7% of the extracts from the walls of *P. denitrificans* and

*P. mucidolens* (corresponding results were not computed for other species but were apparently similar). Muramic acid, 2,6-diaminopimelic acid, galactosamine, and unknowns I to VI were absent from all lipids, and glucosamine was detected only as a trace component (1.8% of the total wall glucosamine) in the extract from *P. taetrolens.* A small amount of an unidentified ninhydrin-positive component, partly overlapping phenylalanine on a chromatogram from the AutoAnalyzer, was found for *P. chlororaphis;* this component had not been detected in hydrolysates of whole walls.

The major amino components of the lipids were ethanolamine and ornithine. The results of quantitative analyses for ornithine and for phosphorus are included in Table 5 (accurate analyses for ethanolamine were not possible by using the AutoAnalyzer, as the peak for this component was usually superimposed on a sharply rising baseline; estimates for ethanolamine contents of whole walls are given in Table 3). Lipid ornithine accounted for almost all (90 to 99%) of the ornithine present in 7 of the 10 species of wall for which this calculation could be made (appropriate analyses were not available for *P. stutzeri* and *P. syncyanea*). Only for *P. aureofaciens* was the value (64%) less than 80%, but, as these walls had the lowest ornithine content, experimental error and the proportion of ornithine from other sources could be greatest in this case. Except for *P. stutzeri* and *P. syncyanea,* the lipids had surprisingly low phosphorus analyses, showing that they contained a high proportion of nonphospholipids. Lipid phosphorus as a percentage of the total phosphorus ranged from 5% (*P. aureofaciens*) to 46% (*P. stutzeri*).

The composition of lipid samples was studied by means of thin-layer chromatography. Typical chromatograms are shown in Fig. 1 and 2. Cardiolipin, phosphatidylethanolamine, and phosphatidylglycerol were present in all lipids, whereas small amounts of phosphatidylcholine were probably present in lipids from *P. mucidolens, P. fluorescens,* and *P. synxantha.* The spots thought to correspond to phosphatidylcholine (Fig. 1) were the same shape as those of the reference compound and reacted with reagents for phosphorus and with the Dragendorff reagent, but not with ninhydrin. The amounts of the phospholipid were too small for choline to be detected by paper chromatography of acid hydrolysates corresponding to 1 mg of total lipids. The major ninhydrin-positive spot located just behind phosphatidylethanolamine on all chromatograms had the properties of the ornithine-containing lipid previously isolated from *P. rubescens* and detected in small amount in *P. maltophilia* (53). This component almost certainly accounts for most or all of the ornithine present in the mixed lipids. Unidentified ninhydrin-positive compounds were present in the lipids from *P. fragi, P. taetrolens, P. ovalis,* and *P. stutzeri.* One of these lipids (present only in *P. taetrolens*) was a phospholipid with a mobility similar to that of phosphatidylcholine (Fig. 1). A second lipid (present in *P. taetrolens, P. fragi,* and *P. stutzeri*) was also a phospholipid: it moved slightly ahead of lysophosphatidylethanolamine using either neutral or basic solvent systems. The two closely spaced spots with mobilities slightly less than that of lysophosphatidylethanolamine (Fig. 1), found for *P. taetrolens* and *P. ovalis,* reacted with ninhydrin but did not contain phosphorus. No indication as to the identities of the amino components of these ninhydrin-positive lipids was provided by quantitative analysis of the lipid mixtures.

TABLE 5. *Analyses of crude lipid extracted from cell walls*[a]

| Species | Lipid content | Phosphorus | Ornithine |
|---|---|---|---|
| *Pseudomonas aureofaciens* | 16.2 | 0.36 | 1.74 |
| *P. chlororaphis* | 16.9 | 0.66 | 3.89 |
| *P. denitrificans* | 20.9 | 0.89 | 6.71 |
| *P. fluorescens* | 22.3 | 1.33 | 6.70 |
| *P. fragi* | 24.5 | 1.44 | 3.33 |
| *P. mucidolens* | 23.0 | 1.77 | 5.10 |
| *P. ovalis* | 29.1 | 1.73 | 4.83 |
| *P. putida* | 20.2 | 1.43 | 5.10 |
| *P. stutzeri*[b] | 25.3 | 3.11 | (2.83) |
| | (24.4) | (2.86) | |
| *P. syncyanea*[b] | 40.7 | 2.47 | (3.40) |
| | (40.0) | (2.35) | |
| *P. synxantha* | 24.4 | 0.85 | 5.96 |
| *P. taetrolens* | 19.1 | 1.90 | 4.86 |

[a] Results for lipid content are given as percentage (dry weight) of cell wall, other results are percentage (dry weight) of lipid extract.

[b] Results in parentheses are for batches of walls prepared by using 1-min disintegration of cells.

The presence of streaked components behind phosphatidylglycerol on chromatograms for most lipids (Fig. 1) suggested the occurrence of glycolipids containing hexuronic acids (52, 54). The suspicion was confirmed by chromatography using an acidic solvent system (Fig. 2), which is particularly useful for the detection of monohexuronosyldiglycerides. With this system, these glycolipids run to a position between cardiolipin and phosphatidylethanolamine (occupied by free fatty acids in a neutral solvent system), and are found as discrete spots instead of streaks. Spots corresponding to the acidic glycolipid were found

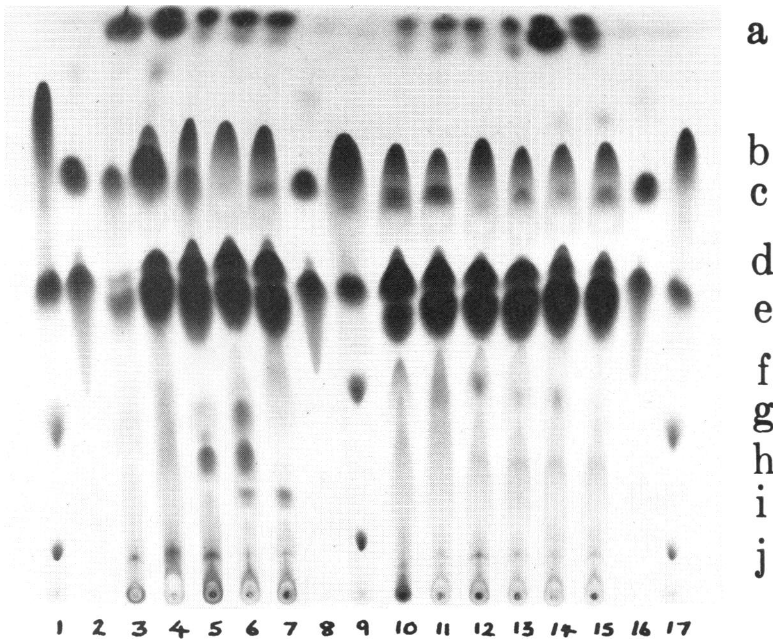


FIG. 1. *Thin-layer chromatogram of lipids from cell walls. Chromatogram run on silica gel G, developed with chloroform-methanol-water (65:25:4, v/v), spots detected by $I_2$. 1, 9, 17: Cardiolipin, phosphatidylethanolamine, phosphatidylcholine, and lysophosphatidylcholine; 2, 8, 16: oleic acid and phosphatidylethanolamine (synthetic, containing a trace of lysophosphatidylethanolamine); 3: P. aureofaciens; 4: P. chlororaphis; 5: P. fragi; 6: P. taetrolens; 7: P. ovalis; 10: P. syncyanea; 11: P. putida; 12: P. mucidolens; 13: P. fluorescens; 14: P. synxantha; 15: P. denitrificans. a, Nonpolar lipids; b, cardiolipin; c, fatty acid; d, phosphatidylethanolamine; e, ornithine-containing lipid; f, phosphatidylglycerol; g, phosphatidylcholine; h, hexuronosyldiglyceride; i, lysophosphatidylethanolamine; j, lysophosphatidylcholine.*

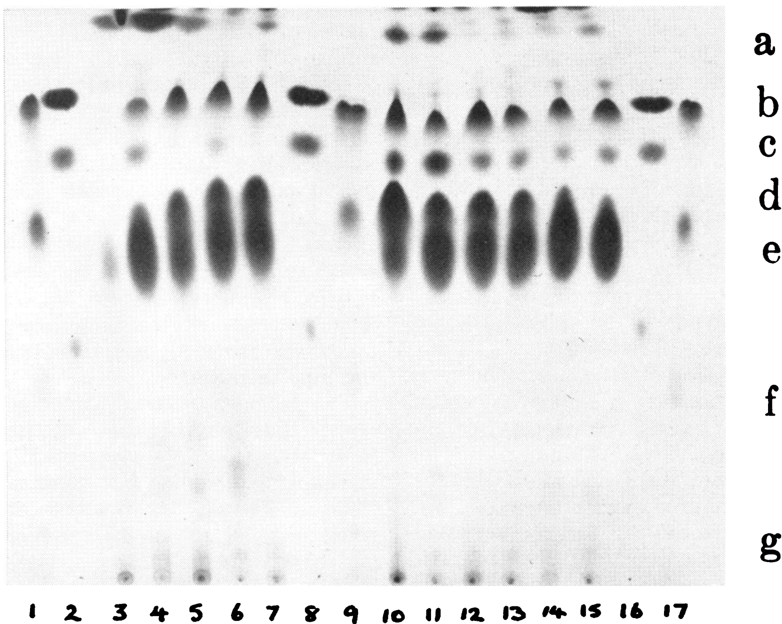


FIG. 2. *Thin-layer chromatogram of lipids from cell walls. Chromatogram run on silica gel G, developed with chloroform-methanol-acetic acid-water (85:15:10:4, v/v), spots detected by $I_2$. 2, 8, 16: Glycolipids from P. diminuta (reference 54); 1, 3–7, 9–15, 17: as in Fig. 1. a, Fatty acid; b, cardiolipin and glucosyldiglyceride; c, hexuronosyldiglyceride; d, phosphatidylethanolamine; e, ornithine-containing lipid; f, phosphatidylcholine; g, lysophosphatidylcholine.*

for all species except *P. stutzeri*, *P. fragi*, and *P. ovalis* (its detection for *P. aureofaciens* required the use of larger amounts of lipid than were used on chromatograms shown here). Glucuronic acid and its lactone were identified by paper chromatography of acid hydrolysates of the lipids from *P. putida* and *P. syncyanea*, which seemed to have the highest contents of glycolipids.

The only other lipid components identified were free fatty acids. They occurred in the greatest proportions in *P. aureofaciens* and *P. chlororaphis*. Unidentified nonpolar lipids must be major components for *P. aureofaciens*, which had the lowest contents of lipid phosphorus and ornithine. Spots of the mixed lipids from this species on chromatograms were only slowly penetrated by the solvents used, and spots tailing back from the solvent front were observed on developed chromatograms (Fig. 1, 2). The lipids from *P. stutzeri* were bright yellow and probably contained the pigment noted in the whole walls.

Because of the striking similarities in the lipids of the different bacterial species, the lipids present in the growth medium were examined. The content of crude "lipids" of Nutrient Agar was 6.5%; after purification by washing, the lipid content was reduced to 0.095%. The pattern of components detected was quite different from that of the bacterial lipids and, when a similar loading for each was used, only a trace of material in about the position of the ornithine-containing lipid was found for the lipids from Nutrient Agar.

**Fatty acid composition of loosely bound lipids.** The fatty acid compositions of all bacterial lipids were qualitatively similar, even to the presence of the same minor and trace components. The acids identified and the spread of analytical data are given in Table 6. Straight-chain saturated $C_{16}$ and monoenoic $C_{16}$ and $C_{18}$ acids were the major components in all cases. Minor acids were present in the greatest amount (26.5% of total acids) in the lipids of *P. taetrolens*. Apart from small amounts of saturated, straight-chain $C_{12}$, $C_{14}$, $C_{15}$, and $C_{18}$ acids, these components could not be identified by gas-liquid chromatography with the aid of available reference compounds. The components were not detected in unmethylated lipids, in a reagent-solvent blank, or in methyl esters from lipids of Nutrient Agar. The major acids in the latter lipids were straight-chain saturated $C_{16}$ (44.8%) and $C_{18}$ (15.6%) and monoenoic $C_{16}$ (3.7%) and $C_{18}$ (29.0%) acids. The major, unidentified, bacterial acids had the following carbon numbers on a column of polydiethylene glycol succinate at 180 C: A, 17.47; B, 19.00; C, 19.42; D, 20.00; E, 21.00. The carbon numbers were apparently less when using a column of Apiezon L at 220 C; for the principal unknowns

TABLE 6. *Fatty acid composition of loosely bound lipids of cell walls*

| Fatty acid[a] | Range[b] |
|---|---|
| 14:0 | 0.2–1.2 |
| 15:0 | Trace–4.3 |
| 16:0 | 24.9–53.9 |
| 16:1 | 11.8–39.1 |
| Unknown A | 0.2–7.6 |
| 18:0 | 0.4–1.8 |
| 18:1 | 12.4–42.8 |
| Unknown B | Trace–6.9 |
| Unknown C | Trace–3.7 |
| Other acids | Trace–7.4 |

[a] Number to left of colon is number of carbon atoms; number to right is number of double bonds.

[b] Ranges of results, expressed as percentage of total fatty acid, obtained for the 12 species studied.

A and B they were 16.77 and 17.36, respectively. Only peaks corresponding to the unsaturated $C_{16}$ and $C_{18}$ acids were lost after bromination of the mixed methyl esters. The chromatographic data therefore rule out simple saturated and unsaturated alkanoic acids; straight-chain 2- and 3-hydroxy acids were also eliminated. However, the lower carbon numbers of the unidentified acids on the nonpolar column indicate that these acids are relatively polar, and it should be noted that hydroxy acids occur in the phosphatidylethanolamine and nonphospholipids containing lysine or ornithine from *Streptomyces sioyaensis* (29, 31).

**Extraction of cell walls with EDTA.** Walls were treated with EDTA at *p*H 9.2 under conditions used with other pseudomonads (21, 53), and wall-free supernatant fluids were analyzed for phosphorus. The results of these experiments are given in Table 7. For most species, a small but definite extraction of phosphorus by EDTA was found. Only for *P. aureofaciens* was the extent of extraction comparable with that found for *P. aeruginosa* and *P. alcaligenes* (21); the extraction of phosphorus by borate buffer alone was also exceptionally high for *P. aureofaciens*. The sensitivity to EDTA of walls from *P. aeruginosa* grown on Tryptone Glucose Extract Agar was identical with that of similar walls studied previously (21), and was about twice that of walls from organisms grown on Nutrient Agar (Table 7). The latter walls had the lower content of phosphorus (1.90% versus 2.18%) and the higher content of carbohydrate (10.5% versus 7.4%).

## DISCUSSION

The walls of each of the species studied contain protein, carbohydrate, peptidoglycan, and loosely

TABLE 7. *Extraction of cell-wall phosphorus by EDTA*[a]

| Species | EDTA in buffer | Buffer alone |
|---|---|---|
| *Pseudomonas aeruginosa*[b] | 20.0 | 5.8 |
| *P. aeruginosa*[c] | 40.4 | 11.2 |
| *P. aureofaciens* | 44.8 | 25.5 |
| *P. chlororaphis* | 18.2 | 11.9 |
| *P. denitrificans* | 10.2 | 5.8 |
| *P. fluorescens* | 9.3 | 7.9 |
| *P. fragi* | 10.0 | 7.6 |
| *P. mucidolens* | 6.7 | 6.6 |
| *P. ovalis* | 6.0 | 3.9 |
| *P. putida* | 10.4 | 4.7 |
| *P. stutzeri* | 4.5 | 5.1 |
| *P. syncyanea* | 18.3 | 16.3 |
| *P. synxantha* | 14.3 | 9.0 |
| *P. taetrolens* | 9.5 | 5.9 |

[a] Results expressed as percentage of total phosphorus extracted on treatment of walls for 1 hr at 20 C with 3.4 mM EDTA in borate buffer (*p*H 9.2) or with buffer alone.

[b] Walls from cells grown on Nutrient Agar.

[c] Walls from cells grown on Tryptone Glucose Extract Agar.

bound lipid. Although no attempt was made to isolate lipopolysaccharide, the analytical results (e.g., for aldoheptose, 2-keto-3-deoxyoctonic acid, glucosamine, and phosphorus not in loosely bound lipid) indicate that lipopolysaccharide was also present in all species of wall. Except in *P. syncyanea*, protein (30 to 35%) was probably the major component of the wall. In *P. syncyanea*, the content of protein (about 22%) was substantially less than that of loosely bound lipid (about 40%). The walls of this organism also contained less peptidoglycan (judged from analyses for muramic acid and 2,6-diaminopimelic acid) than did the other walls. It seems likely that the extensive comminution of the walls during disintegration of cells of *P. syncyanea* is related to the unusual balance of their components. The similar fragility of the walls of *P. stutzeri* seems to require a different explanation, although here again the content of muramic acid was low and the total carbohydrate was less than that of all other walls. For the remaining species, the amounts of peptidoglycan were comparable with those in *P. diminuta* and *P. maltophilia*, more than that in *P. rubescens*, and considerably less than that in *P. pavonacea* (53).

Perhaps the most interesting results obtained in the survey concern the unidentified, ninhydrin-positive components (unknowns I to VI), believed to be amino sugars, and the loosely bound lipids. The most widely distributed unknown was IV (quinovosamine?), which apparently occurred in six species. Fucosamine (unknown VI?) is present in the lipopolysaccharide of *P. aeruginosa* (14, 50), and a large variety of other amino sugars occurs in lipopolysaccharides generally (34). The most notable component of the loosely bound lipids was the ornithine-containing lipid which was common to all species. This lipid has been detected in other pseudomonads (53), and a similar or identical lipid has been found in a *Mycobacterium* species (33), *Rhodopseudomonas spheroides* (18), *Rhodospirillum rubrum* (12), *Streptomyces sioyaensis* (30), and *Thiobacillus thiooxidans* (46). Assuming that the lipids found in the present study have an ornithine content of about 20%, similar to that of *P. rubescens* (53), they account for between 9% (*P. aureofaciens*) and 33% (*P. denitrificans* and *P. fluorescens*) of the total lipids. The probable occurrence of hexuronosyldiglycerides in most of the species also serves to link these EDTA-sensitive pseudomonads with EDTA-resistant ones (52–54). A point of difference is that the EDTA-resistant pseudomonads had monoglucosyldiglycerides as their major glycolipids, whereas these compounds were not detected in the present study.

The major phospholipids detected in this study (cardiolipin, phosphatidylethanolamine and phosphatidylglycerol) occur commonly in gram-negative bacteria (e.g., 27, 39). Small amounts of phosphatidylcholine have been detected in some but not all strains of *P. aeruginosa* (3, 19, 24, 39, 43, 47) and in *P. alcaligenes* (32). The ninhydrin-positive phospholipid common to *P. taetrolens*, *P. fragi*, and *P. stutzeri* could be an *O*-ornithyl ester of phosphatidylglycerol, suspected to occur in *P. stutzeri* (26). Although a glucosaminylphosphatidylglycerol has been isolated from *P. ovalis* (37, 38), it was not detected in the present studies. Few (15) suggested that phosphatidylserine was a component of the envelope of *P. denitrificans*. Although this was not confirmed by the present studies, the phosphorus content of the lipids studied by Few (4.3%) was much greater than that of the lipids described here (0.89%). However, the amounts of serine relative to other protein amino acids in most or all of the lipids analyzed were greater than the corresponding relative amounts in the whole walls. Other workers found that serine was the principal amino acid in the fraction of the wall of *P. aeruginosa* which was soluble in methanolic phenol (8), and that a serine-containing phospholipid was associated with lipopolysaccharide from the same organism (25).

The fatty acid compositions of loosely bound lipids were very similar for each species and generally resembled those reported for other pseudomonads (3, 5, 24). Although fatty acid

composition was not studied in the survey of EDTA-resistant pseudomonads (53), the glycolipids of *P. rubescens* contained a significant proportion of odd-numbered and branched-chain acids (52), while saturated $C_{16}$ and monoenoic $C_{18}$ acids constituted about 95% of the total acids in all but one of the lipids of *P. diminuta* (54). Hydroxy acids occur in glycolipids (2, 28) and lipopolysaccharides (14, 22, 23, 41) of *P. aeruginosa*, and in lipids from this organism grown in *n*-hexadecane (43). Such acids were not found, however, in phosphatidylethanolamine from the latter organisms (44) nor in loosely bound lipids from cell walls or whole cells (3, 24). Cyclopropane acids, found in the lipids of *P. aeruginosa* (1, 24), have lower carbon numbers on nonpolar than on polar columns (6) and could account for some of the unidentified acids reported here.

Although all species included in this study were considered sensitive to EDTA, apart from *P. syncyanea* and *P. stutzeri* they seemed to be less sensitive than *P. aeruginosa* and *P. alcaligenes* (51). The present results also suggest that *P. syncyanea* and *P. stutzeri* stand rather apart from the other 10 species and from each other. The chemical basis for sensitivity of these two species may not be strictly comparable with that for the other pseudomonads. As previously noted, the walls of the EDTA-sensitive *P. aeruginosa* and *P. alcaligenes* are relatively rich in phosphorus (21). The walls of the less sensitive pseudomonads contain less phosphorus, but more than those of EDTA-resistant species (53). It may be significant that the walls from *P. aeruginosa* grown on Nutrient Agar had both a lower phosphorus content and a lower sensitivity to EDTA (Table 7) than those from cells grown on Tryptone Glucose Extract Agar. In view of the likely involvement of cation-binding phosphate groups present in lipopolysaccharide, in the action of EDTA (10, 32, 42), a correlation between phosphorus content of walls and sensitivity to EDTA would not be surprising. Analysis of isolated lipopolysaccharides will be necessary to determine whether the correlation is valid and meaningful.

One of the weaknesses of assessing the sensitivity of an organism to EDTA by measuring the bactericidal effect lies in the considerable dependence on the test conditions used (4, 20). Partly for this reason, it is not surprising that EDTA sensitivity determined in this way (51) does not correlate well with that determined by using isolated walls (Table 7), although previous results had encouraged belief in such a correlation (21, 53). However, the failure of EDTA to extract significant amounts of phosphorus from isolated walls need not mean that extraction did not occur with whole cells, as the properties of isolated walls probably do not accurately reflect those of walls in vivo.

Of the pseudomonads included in this study, only *P. fragi*, *P. mucidolens*, *P. stutzeri*, and *P. syncyanea* were not fluorescent organisms, so that they appeared to be a more homogeneous group than the nonfluorescent, EDTA-resistant organisms studied previously. In their classification of aerobic pseudomonads, Stanier, Palleroni, and Doudoroff (49) recognized only two fluorescent species, each divided into biotypes, in addition to *P. aeruginosa*. According to this classification, *P. synxantha*, *P. denitrificans*, *P. chlororaphis*, and *P. aureofaciens* would be included in *P. fluorescens*, and *P. ovalis* would be included in *P. putida*; the position of *P. taetrolens* is not clear from the data available. The results of wall analyses show that *P. synxantha* resembles *P. fluorescens* very closely, but the walls of other species were more distinctive. Before the significance of the various differences in wall composition can be realized, it will be necessary to examine more strains of each species. Nevertheless, it seems likely that the results of such studies could be useful in the classification of pseudomonads, as has proved to be the case for other gram-negative organisms.


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