Lipid Composition and Chemotaxonomy of *Pseudomonas putrefaciens* (*Alteromonas putrefaciens*)

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The major polar lipids in cells of *Pseudomonas putrefaciens* NCIB 10472 grown on nutrient agar were phosphatidylethanolamine, phosphatidylglycerol, a glucosyldiacylglycerol, a glucuronosyldiacylglycerol and an ornithine amide lipid. An additional phospholipid, tentatively identified as acyl phosphatidylglycerol or bis-phosphatidic acid, was a trace component of the wall lipids from broth cultures, which lacked the glycolipids and the ornithine amide lipid. The wall lipids from broth cultures of three further strains of *P. putrefaciens* (NCIB 10471, NCIB 11156 and NCTC 10737) contained all of the above lipids, and in two cases (strains NCIB 10471 and NCIB 11156) had an unusually high content of free fatty acid. Fatty acid compositions of the extractable lipids were qualitatively similar for all four strains: the major components were iso-pentadecanoic acid, pentadecanoic acid, a *cis*-heptadecenoic acid and a *cis*-hexadecenoic acid. Anteiso fatty acids were minor components in strain NCIB 10472. Lipid mixtures in which the ornithine amide lipid was present also contained small amounts of β -hydroxy fatty acids: in strain NCIB 10472 the major ones were the straight-chain and iso-branched C_{16} acids. Lipopoly-saccharides from all four strains had similar, complex fatty acid compositions. The major non-hydroxy acids were the straight-chain and iso-branched C_{13} acids. β -Hydroxy acids common to all strains included the straight-chain C_{11} , C_{12} , C_{13} , C_{14} and C_{15} acids, together with branched-chain C_{13} and C_{15} acids probably belonging to the iso series. The lipopoly-saccharide from strain NCIB 10472 also contained C_{12} and C_{14} hydroxy acids of the same series, and small amounts of C_{13} and C_{15} β -hydroxy acids probably belonging to the anteiso series. The close resemblance in both polar lipid and fatty acid compositions between strains of *P. putrefaciens* and *Pseudomonas rubescens* is further evidence that these species are synonymous. Significant differences between the lipids and fatty acids of *P. putrefaciens* and those reported for a strain of *Alteromonas haloplanktis* do not harmonize with a proposal to transfer the former organism to the genus *Alteromonas*.

INTRODUCTION

In recent years, major advances in the phenotypic characterization of the aerobic pseudomonads and in the elucidation of their phylogenetic relationships have been made, and have led to a more precise, organized and exclusive delineation of the genus *Pseudomonas* (Doudoroff & Palleroni, 1974). One casualty of this process is the organism known as *Pseudomonas putrefaciens*. This organism has attracted attention mainly because of its involvement in the spoilage of protein foods and its occurrence in clinical specimens (e.g. Hugh, 1970; Levin, 1972; Riley *et al.*, 1972; Shewan, 1974; Holmes *et al.*, 1975; Debois *et al.*, 1975; Lee *et al.*, 1977; Owen *et al.*, 1978; Gilardi, 1978). Values reported for the mol % GC of different strains mostly fall outside the range (58 to 70%) accepted for *Pseudomonas* species and vary rather widely: 47.8 to 59.0% was reported by Levin (1972), 44.7 to 54.7% by Lee *et al.* (1977) and 43.6 to 53.2% by Owen *et al.* (1978).

Several investigations have pointed to the existence of two distinct groups of strains within *P. putrefaciens*. The group with a relatively low GC content tends to be weighted with strains of non-clinical origin, and a number of distinctive phenotypic characters (e.g. failure to grow in the presence of 6 to 7% NaCl) have been reported (Levin, 1972; Riley *et al.*, 1972; Holmes *et al.*, 1975; Williams & Levin, 1975; Levin & Van Sickle, 1976). In a recent numerical taxonomic study, Lee *et al.* (1977) were unable to correlate the GC content of a strain with its salt tolerance or other properties, but proposed that *P. putrefaciens* (their phenon E) be transferred to the genus *Alteromonas* as *Alteromonas putrefaciens*, in accord with an earlier suggestion (Gavini & Leclerc, 1974). The genus *Alteromonas* was created (Baumann *et al.*, 1972) precisely to accommodate *Pseudomonas*-like organisms of low GC content (43 to 48%). In another recent study of *P. putrefaciens*, Owen *et al.* (1978) demonstrated the genetic heterogeneity of the species and described four DNA homology groups and their associated phenotypic characteristics. The most homogeneous group of strains (group IV, GC content about 53%) appeared to correspond to the high GC group first noted by Levin (1972), while the strains in group II (GC content $46.1 \pm 1.2\%$) had the properties associated with Levin's low GC group. Owen *et al.* (1978) preferred to retain the name *P. putrefaciens* for all these strains pending a study of DNA relatedness between these organisms and species of *Alteromonas*.

During several of the above studies of *P. putrefaciens*, the close similarity between strains of this species and others named as *Pseudomonas rubescens* became evident (Hugh, 1970; Holmes *et al.*, 1975; Lee *et al.*, 1977; Owen *et al.*, 1978). It was concluded that the two species are synonymous and should be known by the senior synonym *P. putrefaciens*. Further support for this conclusion can be drawn from a comparison of fatty acid compositions. Work in this laboratory on the lipids of *P. rubescens* NCIB 8768 (Wilkinson, 1972; Wilkinson *et al.*, 1973) showed the presence of a complex range of fatty acids (including odd-numbered, branched-chain and hydroxy acids) quite different from those of the 'true' pseudomonads (Moss, 1978). Studies by Dees & Moss (1975) on the total fatty acids from clinical isolates of *P. putrefaciens* showed that the non-hydroxy components were similar to those from *P. rubescens*, and this resemblance has been confirmed by Ikemoto *et al.* (1978). However, Dees & Moss (1975) did not detect any hydroxy acids in *P. putrefaciens*, while Ikemoto *et al.* (1978) found only minor amounts of 3-hydroxy-dodecanoic acid in both *P. putrefaciens* and *P. rubescens*. In contrast, we found β -hydroxy acids (odd-numbered, even-numbered and branched-chain) to be major components both of the lipopolysaccharide (Wilkinson *et al.*, 1973) and of an ornithine amide lipid (Wilkinson, 1972) from *P. rubescens*. Although the latter lipid and also two glycolipids (Wilkinson, 1968*a*) were found only in cells grown on nutrient agar, it seemed unlikely that phenotypic changes in lipid composition could be wholly responsible for the above discrepancy in fatty acid compositions. Partly to resolve this issue, and partly because lipid composition is finding increasing use in bacterial taxonomy (Shaw, 1974; Lechevalier, 1977), it was decided to investigate further both the polar lipid composition and the fatty acid composition of several strains of *P. putrefaciens*.

METHODS

Reference compounds. Phosphatidylethanolamine and lysophosphatidylethanolamine were obtained from Koch-Light, and cardiolipin, phosphatidic acid and phosphatidylglycerol from Sigma. Reference mixtures of fatty methyl esters were obtained from Supelco, Bellefonte, Philadelphia, U.S.A.

Organisms and growth conditions. Four strains of *Pseudomonas putrefaciens* were used: NCIB 10471, NCIB 10472, NCIB 11156 and NCTC 10737. These strains are now distributed by the NCIB as *Alteromonas putrefaciens*. All strains were cultured at 30 °C. Strain NCIB 10472 was grown on nutrient agar (Oxoid) for 24 h, and in nutrient broth (Oxoid CM67) for 18 or 24 h in a 20 l fermenter (Fermentation Design, Durham, Philadelphia, U.S.A.) with aeration at 20 l min⁻¹ and stirring at 300 rev. min⁻¹. Broth cultures were similarly grown for strains NCIB 10471 (16 h), NCIB 11156 (24 h) and NCTC 10737 (24 h). Cells from all broth

cultures were used for the preparation of isolated walls as described previously for *P. rubescens* (Wilkinson *et al.*, 1973).

Extraction of lipids and lipopolysaccharides. Lipids were extracted from freeze-dried whole cells or isolated walls by stirring them for 2 h at room temperature with chloroform/methanol (2:1, v/v), as described previously (Wilkinson *et al.*, 1973). Lipopolysaccharide was obtained from the defatted walls by treatment with hot aqueous phenol, as in previous studies (Wilkinson *et al.*, 1973).

Conditions of hydrolysis and methanolysis. For the release of amino compounds, lipids were hydrolysed with 6.1 M-HCl at 100 °C for 16 h, and acid was removed from the hydrolysates by repeated evaporation over P_2O_5 and KOH. Fatty acids were released from lipids by hydrolysis with 6.1 M-HCl at 100 °C for 4 h, extracted into diethyl ether and esterified by using diazomethane. Fatty methyl esters were also prepared from lipids by mild alkaline methanolysis and by acid methanolysis (Wilkinson, 1972). Fatty acids present in lipopolysaccharides were released by both acid and alkaline hydrolysis (Rietschel *et al.*, 1972). Sugars present in the lipids from whole cells of strain NCIB 10472 were obtained by alkaline methanolysis of the lipids, followed by hydrolysis of the water-soluble deacylation products with 1 M-HCl at 100 °C for 1 h, and careful neutralization of the hydrolysate with Dowex 1 resin (HCO_3^- form).

Thin-layer chromatography (t.l.c.). Chromatograms were run on ready-made plates of silica gel 60 F₂₅₄ (Merck) and on plates of silica gel G (with or without 5% $AgNO_3$) prepared in the laboratory. Polar lipids were separated by using the following solvent systems: A, chloroform/methanol/water (65:25:4, by vol.); B, chloroform/methanol/7 M-ammonia (65:25:4, by vol.); C, chloroform/methanol/acetic acid/water 85:15:10:4, by vol.); D, methyl acetate/propan-1-ol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.; Vitiello & Zanetta, 1978). Dichloromethane was used as the solvent for the chromatography of fatty methyl esters; bands were detected by using ethanolic 2',7'-dichlorofluorescein, and the separated esters were recovered by elution with diethyl ether. Reagents used for the detection of other compounds were those described by Wilkinson (1972), with the addition of α -naphthol for the detection of glycolipids (Jacin & Mishkin, 1965).

Paper chromatography and electrophoresis. Separations were carried out on Whatman no. 1 paper. Solvent systems used were as follows: E, ethyl acetate/pyridine/water (5:2:5, by vol.; upper phase); F, propan-1-ol/18 M-ammonia/water (6:3:1, by vol.); G, propan-2-ol/18 M-ammonia/water (7:1:2, by vol.); H, butan-2-ol/88% formic acid/water (15:3:2, by vol.); I, phenol/18 M-ammonia/water (80:1:20, by vol.). Buffer systems used were as follows: J, pyridine/acetic acid/water (5:2:43, by vol.) pH 5.3; K, pyridine/acetic acid/water (1:10:89, by vol.) pH 3.6; L, buffer system K adjusted to pH 2.7 with formic acid (Kosakai & Yosizawa, 1975). Detection reagents used were ninhydrin, alkaline $AgNO_3$, aniline hydrogen oxalate, the Hanes/Isherwood reagent for phosphates, and periodate/Schiff reagents as in previous studies (Wilkinson, 1968a; Wilkinson *et al.*, 1973).

Gas-liquid chromatography (g.l.c.). Fatty methyl esters were separated on a column (3.8 m $\times$ 2 mm) packed with 10% (w/w) diethylene glycol succinate on Chromosorb W (80 to 100 mesh), operated at 165 °C with a flow rate (N_2) of 50 ml min⁻¹. Peak areas were determined by using a Supergrator-2 computing integrator (Columbia Scientific Industries, Austin, U.S.A.). Combined gas-liquid chromatography/mass spectrometry (g.l.c./m.s.) of the esters was carried out at the Physico-Chemical Measurements Unit, Aldermaston, Berks. In some instances, β -hydroxy esters were converted into their *O*-trimethylsilyl ethers prior to chromatography (Eglinton *et al.*, 1968).

Other analytical methods. Total carbohydrate was determined by the phenol/ H_2SO_4 method (Dubois *et al.*, 1956), glucuronic acid by the method of Blumenkrantz & Asboe-Hansen (1973) and D-glucose enzymically by using the GOD-Perid reagent (Boehringer). Amino compounds were identified by using a Locarte autoanalyser.

RESULTS

Polar lipid compositions

To determine whether *P. putrefaciens* was able to produce the glycolipids and ornithine amide lipid found in *P. rubescens* (Wilkinson, 1968a, 1972), the unbound lipids were extracted from whole cells of strain NCIB 10472 grown on nutrient agar. T.l.c. of the lipids in solvent systems A, B and C, with the aid of reference phospholipids and several methods of detection, established the presence of phosphatidylethanolamine (the major phospholipid), phosphatidylglycerol and traces of both lysophosphatidylethanolamine and an unidentified phospholipid which migrated only slightly from the origin. Also detected were spots with the mobilities and reactions expected for the ornithine amide lipid, a glucosyldiacylglycerol and a glucuronosyldiacylglycerol. The presence of ethanolamine and

ornithine in acid hydrolysates was determined by autoanalysis, paper electrophoresis in buffer system J, and two-dimensional paper chromatography in solvent systems H and I. Analysis of the water-soluble deacylation products gave a value of 2% (expressed as glucose) for the carbohydrate content of the lipid. The monosaccharide components were identified as D-glucose (by paper chromatography in solvent system E and a positive reaction with glucose oxidase) and glucuronic acid (by colorimetric analysis and paper electrophoresis in buffer system L), in approximately equal amounts. Thus, the polar lipid composition of agar-grown *P. putrefaciens* NCIB 10472 closely resembles that determined for corresponding cultures of *P. rubescens* NCIB 8768 (Wilkinson, 1968*a, b*, 1972).

For comparison with the above data, unbound lipids were also extracted from the isolated walls of cells of strain NCIB 10472 grown in nutrient broth. The phospholipid composition was similar to that of agar-grown cells, but the proportion of phosphatidylglycerol was less and there was a trace of an additional phospholipid with a mobility similar to that of cardiolipin on t.l.c. in solvent system A. Other differences were the absence of the ornithine amide lipid and both glycolipids, and the presence of a moderate amount of free fatty acid. Except for the acquisition of the additional phospholipid, this change in lipid composition for *P. putrefaciens* closely paralleled that observed for *P. rubescens* (Wilkinson *et al.*, 1973). The change is unlikely to be due to the use of isolated walls rather than whole cells in the case of the broth culture, as in the earlier work on *P. rubescens* both types of material had the same complement of lipids.

For the remaining strains of *P. putrefaciens*, only the wall lipids from broth cultures were examined. Somewhat unexpectedly, the results were a composite of those obtained for strain NCIB 10472 grown under the two conditions. Thus, all mixtures contained phosphatidylethanolamine, phosphatidylglycerol, traces of lysophosphatidylethanolamine and the two unidentified phospholipids of high and low mobility respectively, both glycolipids, the ornithine amide lipid and free fatty acid. The proportions of the glycolipids were highest for strains NCIB 10471 and NCIB 11156, both of which also contained substantial amounts of free fatty acid (probably the major polar lipid in these strains). The proportion of ornithine amide lipid was highest in strain NCIB 10471, while the amount of unidentified high-mobility phospholipid in this strain was extremely small. Further attempts were made to identify this phospholipid using the extracts from strains NCIB 10472 and NCTC 10737. A small amount of partially purified material from the latter strain was obtained and compared with reference cardiolipin and phosphatidic acid by t.l.c. and co-chromatography. The unknown was clearly distinguished from both reference compounds. Using plates of silica gel 60 F₂₅₄ and solvent systems A or D, the unknown had a mobility approximately twice that of either reference compound (both of which were less mobile than phosphatidylethanolamine). On silica gel G with solvent system A, the unknown occupied a position between phosphatidylethanolamine and cardiolipin (the most mobile phospholipid).

Evidence that the unidentified phospholipid was a derivative of phosphatidylglycerol came from the study of lipid deacylation products. On paper chromatography (solvent systems F and G) and electrophoresis (buffer systems J and K), the only phosphate esters detected among the products from the total lipids from strains NCIB 10472 and NCTC 10737 were those expected from phosphatidylethanolamine and phosphatidylglycerol. Paper electrophoresis (buffer system J) of the deacylated, partially purified unknown indicated that the product was glycerophosphorylglycerol. This result, and the t.l.c. data, suggest that the parent phospholipid was either acyl phosphatidylglycerol or bis-phosphatidic acid.

Fatty acid compositions

The qualitative differences in polar lipid composition between cells of strain NCIB 10472 grown on nutrient agar and walls from the broth culture prompted a comparison of the respective fatty acid compositions. Results for the ester-bound fatty acids obtained by

Table 1. *Non-hydroxy fatty acid composition of the readily extracted lipids from whole cells and isolated walls of Pseudomonas putrefaciens NCIB 10472*

Lipids were extracted from whole cells (grown on nutrient agar at 30 °C for 24 h) and isolated walls (from cells grown in nutrient broth at 30 °C for 18 h) with chloroform/methanol (2:1, v/v). Fatty methyl esters were prepared by mild alkaline methanolysis or, in the case of the results in parentheses, by hydrolysis at 100 °C for 4 h with 6.1 M-HCl followed by treatment of the fatty acids with diazomethane. Fatty esters were determined by g.l.c.

Fatty acid*	Relative retention time	Percentage of total peak area	
		Cell lipids	Wall lipids
i-13:0	0.33	1.0 (1.2)	3.0
i-14:0	0.45	10.6 (11.7)	5.7
14:0	0.52	1.1 (1.1)	1.3
i-15:0	0.63	10.6 (11.9)	30.7
a-15:0	0.65	4.1 (4.1)	0.7
15:0	0.72	2.3 (2.3)	4.3
15:1	0.82	trace (trace)	1.2
i-16:0	0.85	7.8 (9.4)	2.0
16:0	1.00	7.0 (7.7)	6.3
16:1	1.17	26.6 (25.6)	19.1
a-17:0	1.27	0.8 (trace)	0.1
17:0	1.39	1.8 (1.1)	2.7
17:1	1.59	11.9 (10.5)	16.1
18:0	1.95	1.2 (0.5)	0.4
18:1	2.23	11.7 (11.5)	5.0
19:0	2.72	trace (trace)	trace
19:1	3.07	1.5 (1.2)	1.4

* In fatty acid designations, the first number indicates the number of carbon atoms and the second the number of double bonds; the prefixes indicate iso (i) or anteiso (a) branching.

mild alkaline methanolysis are given in Table 1. Only non-hydroxy fatty acids were detected. These ranged from C₁₃ to C₁₉ acids and included saturated, monoenoic, branched-chain and straight-chain components. Although minor amounts of two anteiso acids were detected (particularly in the whole-cell lipids), branched-chain components mainly belonged to the iso series. As noted in earlier studies of the non-hydroxy fatty acids from *P. putrefaciens* and *P. rubescens*, C₁₅ and C₁₇ make up unusually high proportions of the totals. Quantitative differences in fatty acid composition between the whole-cell lipids and the wall lipids, although substantial, are less striking than the differences in polar lipid composition. Similar quantitative differences in fatty acid composition have been observed between cells of *P. rubescens* grown under different conditions, and between the individual polar lipids in the same batch of cells (Wilkinson, 1972; Wilkinson *et al.*, 1973).

To check whether the fatty acid composition determined by alkaline transesterification also represented the total fatty acid composition, fatty acids were released from the whole-cell lipids of strain NCIB 10472 by acid hydrolysis. Although the non-hydroxy acids released by the two methods were similar (Table 1), traces of additional fatty methyl esters with long retention times were present in the mixture obtained from the acid hydrolysate. These additional components were isolated by preparative t.l.c. on silica gel G, on which they had the mobility of β -hydroxy methyl esters. The retention times on g.l.c. indicated that all but one of the esters belonged to two homologous series, one of which contained the C₁₄ to C₁₈, straight-chain, β -hydroxy esters. The non-hydroxy fatty acid composition of the lipids suggested that the second series contained the corresponding iso-branched β -hydroxy esters, and that the remaining ester was anteiso-branched. Confirmation of the β -hydroxy group and the carbon number of each of the esters was obtained by g.l.c./m.s. The mass spectra contained the expected base peak at m/e 103 and the diagnostic peaks at M-18, M-50 and M-92. Similarly, the mass spectra of the

Table 2. *Hydroxy fatty acid composition of the readily extracted lipids from whole cells of Pseudomonas putrefaciens* NCIB 10472

The total fatty acids in lipids extracted from cells grown on nutrient agar were released by acid hydrolysis and esterified as described in Table 1. The hydroxy ester fraction was isolated by preparative t.l.c. and analysed by g.l.c.

Fatty acid*	Relative retention time	Percentage of total peak area
3-OH i-14:0	0.47	2.5
3-OH 14:0	0.54	2.4
3-OH i-15:0	0.63	5.2
3-OH 15:0	0.74	5.1
3-OH i-16:0	0.86	33.8
3-OH 16:0	1.00	32.9
3-OH i-17:0	1.16	3.3
3-OH a-17:0	1.24	3.1
3-OH 17:0	1.35	6.9
3-OH i-18:0	1.58	4.8
3-OH 18:0	1.84	trace

* The prefix 3-OH indicates a β -hydroxy group; other designations are as indicated in Table 1.

O-trimethylsilyl derivatives of the esters contained the expected peaks at m/e 175, $M-15$, $M-31$, $M-47$ and $M-73$ (Eglinton *et al.*, 1968). Quantitative data for the hydroxy fatty acid composition of the whole-cell lipids is given in Table 2. By analogy with the results obtained for *P. rubescens* (Wilkinson, 1972), these hydroxy acids are expected to be amide-bound components of the ornithine-containing lipid. In this lipid from *P. rubescens*, the major β -hydroxy acids were the straight-chain C_{16} acid and, apparently, branched-chain C_{17} and C_{19} acids.

Although hydroxy acids were absent from the wall lipids of broth-grown cells of strain NCIB 10472 (consistent with the absence of the ornithine amide lipid), they were expected to be present in the wall lipopolysaccharide. The results for fatty acid composition (Table 3), determined after acid or alkaline hydrolysis of the lipopolysaccharide, bear out this expectation. As in the case of hydroxy acids from the ornithine amide lipid, both straight-chain and branched-chain compounds were detected, and the main series of branched-chain compounds was again assumed to contain the iso acids. The complexity of the fatty acid mixtures (augmented by dehydration of the β -hydroxy acids) was such that g.l.c. alone was insufficient for the resolution and identification of all components. The acids listed in Table 3 were identified by the retention times of their methyl esters, the separation of hydroxy and non-hydroxy esters by t.l.c., and the examination of each fraction by g.l.c./m.s. Whereas the iso- C_{15} acid was the most abundant in the readily extracted wall lipids (Table 1), the iso- C_{13} acid was the major non-hydroxy acid in the lipopolysaccharide. Similarly, the hydroxy acids in the lipopolysaccharide were of shorter chain length than those in the ornithine amide lipid (Table 2). Qualitatively, the fatty acid composition of the lipopolysaccharide from *P. putrefaciens* strain NCIB 10472 was similar to that determined for *P. rubescens* NCIB 8768 (Wilkinson *et al.*, 1973). The major differences were in the hydroxy acid components: the lipopolysaccharide from *P. rubescens* did not contain the acids to which the anteiso structure has been assigned, nor the components considered to be the iso- C_{12} and iso- C_{14} acids.

To determine whether the fatty acid compositions determined for strain NCIB 10472 were typical for *P. putrefaciens*, the corresponding data were obtained for the wall lipids and lipopolysaccharides from strains NCIB 10471, NCTC 10737 and NCIB 11156. The results are given in Tables 4 and 5, along with those obtained for a second batch of strain NCIB 10472 (grown for 24 h instead of 18 h) and those for *P. rubescens* NCIB 8768. For

Table 3. Fatty acid composition of lipopolysaccharide from *Pseudomonas putrefaciens* NCIB 10472

Lipopolysaccharide was isolated from the defatted walls of cells grown in nutrient broth at 30 °C for 18 h (Table 1). Fatty acids were released by hydrolysis at 100 °C for 5 h with 4 M-HCl (A) or 4 M-NaOH (B). The methyl esters were prepared using diazomethane and were estimated by g.l.c.

Fatty acid*	Relative retention time	Percentage of total peak area	
		A	B
11:0	0.11	0.3	0.3
i-12:0	0.13	trace	trace
12:0	0.14	5.1	5.9
i-13:0	0.17	23.9	22.9
<i>13:0</i>	0.19	3.5	5.1
i-12:1			
<i>i-14:0</i>	0.23	3.8	5.1
12:1			
14:0	0.27	2.0	6.0
i-13:1			
<i>i-15:0</i>	0.31	1.6	2.4
13:1			
15:0	0.36	0.9	0.9
<i>i-14:1</i>			
3-OH i-10:0	0.43	1.5	0.6
<i>14:1</i>			
i-16:0			
3-OH 10:0			
16:0	0.53	1.7	0.7
<i>i-15:1</i>			
3-OH i-11:0	0.56	0.5	0.2
3-OH a-11:0	0.62	2.2	1.7
3-OH 11:0	0.72	3.5	3.6
3-OH i-12:0	0.85	6.6	5.2
3-OH 12:0	1.00	14.6	13.0
3-OH i-13:0	1.07	trace	trace
3-OH a-13:0	1.18	6.6	4.5
3-OH 13:0	1.38	4.9	4.4
3-OH i-14:0	1.63	8.8	7.0
3-OH 14:0	1.92	5.5	6.8
3-OH i-15:0	2.05	1.7	1.9
3-OH a-15:0	2.26	0.7	0.7

* Fatty acid designations are as indicated in Tables 1 and 2. Braces indicate that the methyl esters of the acids were not resolved on the column used; the major components of the mixtures are italicized.

wall lipids, fatty methyl esters were prepared by three different methods. Mild alkaline methanolysis was used to determine only those acids that were ester-bound (columns A in Table 4), acid methanolysis was used to determine the total of free and ester-bound non-hydroxy acids (columns B in Table 4), and acid hydrolysis was used to ensure the release of amide-bound acids (columns C in Table 4 give the data for non-hydroxy acids present in the hydrolysates). The range of non-hydroxy fatty acids was the same for all four strains of *P. putrefaciens*, except that anteiso acids were only detected for strain NCIB 10472. In all strains the major fatty acids were the iso-C₁₅ acid, pentadecanoic acid and monoenoic C₁₆ and C₁₇ acids. Argentation t.l.c. indicated that both unsaturated acids were *cis*-isomers. Only strain NCIB 10472 contained a substantial proportion of the iso-C₁₄ acid. The results

Table 4. Comparison of the non-hydroxy fatty acid compositions of the readily extracted lipids from isolated walls of various strains of *Pseudomonas putrefaciens*

Lipids were extracted from the isolated walls of cells grown in nutrient broth at 30 °C for 16 h (strain NCIB 10471) or 24 h (other strains). Fatty methyl esters were prepared by alkaline methanolysis (A), acid methanolysis (B) or acid hydrolysis followed by treatment of the fatty acids with diazomethane (C). Fatty esters were determined by g.l.c. Data for the wall lipids of *Pseudomonas rubescens* NCIB 8768 were obtained by BF_3 -catalysed methanolysis by Wilkinson *et al.* (1973). +, Present but not determined; —, not detected.

Fatty acid*	Strain ...	Percentage of total peak area											
		NCIB 10471			NCIB 10472			NCTC 10737			NCIB 11156		
Method ...		A	B	C	A	B	C	A	B	C	A	B	C
i-13:0		2.2	1.1	1.0	3.2	2.6	2.6	4.0	2.7	2.6	0.2	0.1	0.1
i-14:0		0.3	0.2	0.2	8.1	6.9	6.8	1.1	0.9	0.8	0.1	0.2	0.2
14:0		1.3	1.0	0.9	1.6	1.2	1.2	1.3	1.1	1.1	0.9	0.6	0.6
i-15:0		16.0	12.8	12.0	21.6	20.1	20.0	25.1	22.6	22.7	20.4	18.1	18.1
a-15:0		—	—	—	+	3.4	3.3	—	—	—	—	—	—
15:0		21.1	17.7	19.5	9.9	9.9	9.8	12.8	13.4	13.5	13.5	13.9	13.4
15:1		trace	trace	trace	trace	trace	trace	—	—	—	—	—	—
i-16:0		2.7	1.2	1.0	5.1	5.2	4.9	3.9	3.4	3.4	5.8	3.2	3.2
16:0		9.6	8.9	8.4	6.4	5.6	5.4	6.4	6.3	6.3	4.0	4.0	3.8
16:1		11.6	11.9	11.1	17.5	16.7	16.0	14.1	16.0	15.4	15.0	15.0	14.8
a-17:0		—	—	—	trace	trace	trace	—	—	—	—	—	—
17:0		7.4	6.8	6.4	2.8	2.9	3.0	3.3	3.4	3.5	3.5	3.2	3.2
17:1		20.8	30.3	30.5	17.0	20.4	21.2	20.2	23.8	23.8	29.3	36.5	37.1
18:0		0.5	0.5	0.4	0.3	0.3	0.3	1.0	0.7	0.6	0.5	0.2	0.2
18:1		3.7	5.4	5.1	4.3	3.4	4.1	4.9	4.1	4.3	4.0	4.2	4.4
19:0		trace	trace	0.1	0.3	trace	trace	0.1	trace	trace	trace	trace	trace
19:1		1.3	1.4	1.6	1.3	0.8	0.8	1.3	1.1	1.5	1.7	0.6	0.7
Others		1.5	0.8	1.8	0.6	0.6	0.6	0.5	0.5	0.5	1.1	0.2	0.2

* Fatty acid designations are as given in Table 1.

Table 5. Comparison of the fatty acid compositions of lipopolysaccharides from various strains of *Pseudomonas putrefaciens*

Lipopolysaccharides were isolated from the defatted walls of cells grown as described in Table 4. Fatty acids were released by hydrolysis at 100 °C for 5 h with 4 M-HCl, esterified using diazomethane, and estimated by g.l.c. Similar results were obtained after alkaline hydrolysis. Data for the lipopolysaccharide from *Pseudomonas rubescens* NCIB 8768 were obtained by Wilkinson *et al.* (1973). +, Present but not determined; —, not detected.

Fatty acid*	Strain...	Percentage of total peak area				
		NCIB 10471	NCIB 10472	NCTC 10737	NCIB 11156	NCIB 8768
11:0		2.0	0.9	1.0	2.2	—
i-12:0	}	5.0	trace	4.3	1.5	—
Unknown						
12:0		9.1	4.5	6.7	6.0	5.5
i-13:0		16.9	23.5	23.8	15.5	18.9
13:0	}	13.5	7.0	9.3	16.3	9.5
i-12:1						
i-14:0	}	1.1	3.9	0.8	0.8	0.8
12:1						
14:0	}	1.2	1.7	1.9	2.0	1.3
i-13:1						
i-15:0	}	3.8	2.3	2.9	4.9	2.9
13:1						
15:0	}	0.2	1.9	trace	0.2	—
i-14:1						
3-OH i-10:0	}	1.2	2.1	1.8	0.9	2.1
14:1						
i-16:0	}					
3-OH 10:0						
16:0	}	1.1	4.6	1.3	2.1	1.8
i-15:1						
3-OH i-11:0						
3-OH a-11:0		—	+	—	—	—
3-OH 11:0		7.2	4.3	4.2	7.4	7.7
3-OH i-12:0		0.2	4.2	trace	0.3	—
3-OH 12:0		8.3	4.6	5.5	4.8	9.8
3-OH i-13:0		5.2	7.9	9.8	9.4	11.4
3-OH a-13:0		—	1.1	—	—	—
3-OH 13:0		17.9	8.1	15.4	18.3	17.8
3-OH i-14:0		trace	5.1	trace	trace	—
3-OH 14:0		5.2	6.1	8.1	4.0	5.7
3-OH i-15:0		0.7	3.7	2.2	2.5	2.2
3-OH a-15:0		—	1.5	—	—	—
3-OH 15:0		trace	0.8	0.9	0.9	—

* Fatty acid designations and braces have the same significance as in preceding tables. The unknown had a retention time corresponding to i-12:0 but was absent from alkaline hydrolysates.

for the total non-hydroxy acid differed from those for ester-bound acid mainly in the proportion of the C₁₇ monoenoic acid. The difference was greatest for strains NCIB 10471 and NCIB 11156 which, as described above, contained the largest proportions of free fatty acid in the wall lipids. Thus, it appeared that the free fatty acids were enriched in this unusual component. No attempt was made to quantify or rigorously identify the hydroxy acids present in acid hydrolysates. However, all mixtures shown to contain the ornithine amide lipid gave trace components on chromatograms of the fatty methyl esters, of which the retention times indicated that they were C₁₅ to C₁₇ β-hydroxy esters.

The results for the fatty acid compositions of the lipopolysaccharides (Table 5) are qualitatively and semi-quantitatively very similar for all strains. Points of difference again relate mainly to strain NCIB 10472, which was the only one to produce significant amounts of the presumed anteiso, iso-C₁₂ and iso-C₁₄ β -hydroxy acids. Although the quantitative data suggest similarities between certain strains (e.g. NCIB 10471 and NCIB 11156), no reliable conclusions can be drawn on the basis of this evidence. Thus, phenotypic variations in fatty acid composition must occur (and are probably exemplified by the differences observed for the two batches of lipopolysaccharide from strain NCIB 10472, Tables 3 and 5). Also, acid hydrolysis of materials that contain both hydroxy and non-hydroxy fatty acids produces artifacts which may significantly distort the fatty acid composition (Wilkinson, 1974).

DISCUSSION

The results of this study demonstrate that strains of *P. putrefaciens*, like *P. rubescens* NCIB 8768, produce a complex range of β -hydroxy fatty acids, which occur both in lipopolysaccharide and in an ornithine amide lipid (when present). Factors that may have contributed to the failure to recognize this situation in previous studies of the 'total' cellular fatty acids of *P. putrefaciens* are the choice of growth conditions and the method of releasing the fatty acids/esters. Dees & Moss (1975) grew the organisms on Trypticase soy agar, which might be expected to favour the production of the ornithine amide lipid, but used a relatively short time for saponification, which may have been inadequate for the total release of amide-bound fatty acids. Ikemoto *et al.* (1978) used stationary-phase broth cultures, which might not have contained the ornithine amide lipid, and acid methanolysis conditions which were still less drastic than the conditions of acid hydrolysis used in the present work. Relatively low lipopolysaccharide contents for the organisms may also have presented a problem: the yields of lipopolysaccharide were 10% or less of the cell wall for three of the strains used in this study.

The results of this study also reinforce the conclusion that *P. putrefaciens* and *P. rubescens* are synonymous species. Because of phenotypic variation, polar lipid composition is obviously unsatisfactory as a diagnostic or taxonomic character unless the physiological state of the cells is rigorously standardized. However, strains of both species do, under appropriate growth conditions, produce the distinctive glycolipids and the ornithine amide lipid. That surface culture seems to favour their production is probably due to phosphorus limitation of the growth of such cultures (Minnikin & Abdolrahimazadeh, 1976). The persistence of the unusual lipids in broth cultures of three of the strains used in this study may be related to the more luxuriant growth of these compared with the fourth strain (NCIB 10472). The latter strain was also exceptional in the fatty acid compositions of its lipids and lipopolysaccharide, which were even more complex than those of the other strains. Strain NCIB 10472 (ATCC 8072), which was one of the original isolates of *P. putrefaciens* from tainted butter, has also been found to possess atypical properties in other studies of the species. Thus, Levin (1972) found that this was the only 'low GC' strain capable of growth in the presence of 6% NaCl. Owen *et al.* (1978) did not confirm this result, but reported a genome size for the DNA of 1.20 relative to that for the reference strain of DNA homology group II. Strain NCTC 10737, another member of this homology group, had a relative genome size of 1.06. Lee *et al.* (1977) found that strain NCIB 10472 could be differentiated from other strains belonging to their phenon E (which included NCIB 10471, NCIB 11156 and NCIB 8768 – NCTC 10737 was not tested) on nutritional grounds: it failed to grow on any of the compounds tried as a sole source of carbon. However, the main inference that can be drawn from the results of the present study is that the four strains of *P. putrefaciens* and *P. rubescens* NCIB 8768 (all of which are 'low GC' strains) are closely related. No differences which might correlate with the separation (Owen *et al.*, 1978) into DNA homology groups I (which includes strains NCIB 10471

and NCIB 8768) and II (which includes strains NCIB 10472 and NCTC 10737) stand out.

The uncertain taxonomic position of *P. putrefaciens* provided a further stimulus to this study of the polar lipids and fatty acids of this species, since both are potentially useful for the determination of inter-species relationships. Phosphatidylethanolamine and phosphatidylglycerol occur in the majority of Gram-negative bacteria, but glycolipids, ornithine amide lipids and the phospholipid tentatively identified as acyl phosphatidylglycerol or bis-phosphatidic acid are relatively uncommon (Shaw, 1974; Lechevalier, 1977; Finnerty, 1978). However, each of them occurs in diverse organisms. For example, glucosyldiacylglycerols are also found in *Pseudomonas diminuta*, *Pseudomonas vesicularis*, *Yersinia pseudotuberculosis* and *Spirochaeta zuelzerae*, while glucuronosyldiacylglycerols have been isolated from *P. diminuta*, *P. vesicularis*, an unidentified moderately halophilic, halotolerant organism, and a strain from the genus *Actinomyces* (Batrakov & Bergelson, 1978). Organisms that produce an ornithine amide lipid similar or identical to that in *P. putrefaciens* include *Thiobacillus thiooxidans*, *Rhodopseudomonas sphaeroides*, *Desulfovibrio gigas* and *Gluconobacter cerinus*. Strains of several 'true' pseudomonads produce an ornithine amide lipid and a hexuronosyldiacylglycerol when grown on nutrient agar (Wilkinson, 1970). Acyl phosphatidylglycerol is a minor lipid component in strains of *Salmonella typhimurium*, *Escherichia coli*, an *Acinetobacter* species and the marine bacterium *Pseudomonas* BAL-31 (Makula *et al.*, 1978). Evidence for the occurrence of bis-phosphatidic acid in other non-fermentative, rod-shaped marine bacteria has been presented (McAllister & De Siervo, 1975). Nevertheless, the polar lipid compositions determined for *P. putrefaciens* and *P. rubescens* (particularly for surface cultures) are clearly different from those determined for 'true' *Pseudomonas* species in these and other laboratories. Little information on the polar lipid composition of *Alteromonas* species is available for comparison with *P. putrefaciens*. However, cardiolipin (which is absent from *P. putrefaciens*) is a significant phospholipid component of the marine 'pseudomonad' B16 (NCMB 19) which has recently been reclassified as *Alteromonas haloplanktis* (Gordon & MacLeod, 1966; De Siervo & Reynolds, 1975).

The fatty acid compositions determined for the extractable lipids and the lipopolysaccharides of *P. putrefaciens* are even more distinctive. Relatively few Gram-negative bacteria contain significant amounts of branched-chain and odd-numbered fatty acids. Among the extensively studied *Pseudomonas* and related species (Moss, 1978; Ikemoto *et al.*, 1978), only *Pseudomonas maltophilia* (Moss *et al.*, 1973; Moss & Dees, 1975; Tornabene & Peterson, 1978) and *Xanthomonas* species (Rietschel *et al.*, 1975) have similar fatty acid compositions. But although some of the branched-chain hydroxy and non-hydroxy acids in *P. putrefaciens* are also major components of the cellular lipids and/or lipopolysaccharides of *P. maltophilia* and *Xanthomonas* species, significant differences are apparent. Thus, the presence of a heptadecenoic acid is specific to *P. putrefaciens*, and the more extensive range of hydroxy acids produced by this species does not include the α -hydroxy acids reported for the others. *Pseudomonas putrefaciens* is also readily differentiated from *P. maltophilia* by its DNA composition and phenotypic characteristics, including the polar lipid composition (Wilkinson, 1968*b*; Tornabene & Peterson, 1978). No other species for which the fatty acid compositions of the extractable lipids (Lechevalier, 1977) or the lipopolysaccharides (Wilkinson, 1977) have been reported has a range of fatty acids (branched-chain, odd-numbered, hydroxy and non-hydroxy) fully comparable with that for *P. putrefaciens*, and where points of similarity can be found the species bear no obvious close relationship. Apart from the report (Di Rienzo & MacLeod, 1978) that the lipopolysaccharide of *A. haloplanktis* contains mainly C₁₀, C₁₂ and C₁₄ hydroxy acids, there is a dearth of information about *Alteromonas* species. However, the results of the present study indicate that the determination of polar lipid and fatty acid compositions of *Alteromonas* species should assist in the differentiation or reallocation of organisms consigned to this genus.

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