Polar lipids and fatty acids of *Pseudomonas caryophylli*, *Pseudomonas gladioli* and *Pseudomonas pickettii*

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The polar lipids and their fatty acid components in *Pseudomonas caryophylli*, *Pseudomonas gladioli* and *Pseudomonas pickettii* have been identified. In addition to diphosphatidylglycerol and phosphatidylglycerol (a trace only for *P. pickettii*), all three species contained two forms of phosphatidylethanolamine differing in the presence or absence of α -hydroxy fatty acids. This seems to be a distinctive feature of species in *Pseudomonas* RNA homology group II. Also, *P. caryophylli* and *P. gladioli* (but not *P. pickettii*) produced two forms of ornithine amide lipid, differing in the nature (hydroxy or non-hydroxy) of the ester-linked fatty acid. In all three species, the major non-hydroxy acids were hexadecanoic acid, a hexadecenoic acid, an octadecenoic acid, and cyclopropane derivatives of the monoenoic acids. The α -hydroxy acids were the derivatives of the same components, while the amide-linked acid of the ornithine amide lipids was mainly or entirely 3-hydroxyhexadecanoic acid. The possible taxonomic implications of the data are discussed.

Introduction

Homology group II of *Pseudomonas* species, defined on the basis of rRNA–DNA hybridization studies (Palleroni *et al.*, 1973), contains bacteria which are actually or potentially pathogenic to plants or animals. Two subgroups, one containing *Pseudomonas pickettii* and *P. solanacearum*, and the other containing *P. caryophylli*, *P. cepacia*, *P. gladioli*, *P. mallei* and *P. pseudomallei*, were indicated by the results of DNA–DNA hybridization experiments (Palleroni, 1984). Additional members of the homology group have been identified in more recent studies (Byng *et al.*, 1983; De Vos *et al.*, 1985, 1989; Johnson & Palleroni, 1989). The segregation of pseudomonads into five RNA homology groups is consistent with a traditional classification based on phenotypic characters, including numerical analysis thereof (Sneath *et al.*, 1981). Wider phylogenetic considerations have led to the placement of homology group II species in the rRNA superfamily III of Proteobacteria (De Vos *et al.*, 1989) or subgroup β -2 of the purple bacteria (Woese *et al.*, 1984; Woese, 1987).

Earlier studies (Kawai *et al.*, 1988; Cox and Wilkinson, 1989) have shown that *P. cepacia* produces an unusual range of polar lipids. Specifically, these include

two forms of phosphatidylethanolamine (PE) and two ornithine amide lipids. In each pair of lipids, one member has an α -hydroxy fatty acid in place of the more usual non-hydroxy acid. The same two forms of PE have been described for *P. solanacearum* (Drigues *et al.*, 1981), while α -hydroxy fatty acids have been detected in studies of the total cellular fatty acids from other species belonging to homology group II (Moss, 1978; Oyaizu & Komagata, 1983; Dees *et al.*, 1983; Christenson *et al.*, 1989; Veys *et al.*, 1989). As both polar lipids and fatty acids have often proved to be useful chemotaxonomic markers in bacteria (Ratledge & Wilkinson, 1988), it was of interest to extend the study of these components to other group II pseudomonads.

Methods

Organisms and culture conditions. The following type strains were used: *P. caryophylli* NCPPB 2151, *P. gladioli* NCPPB 1891, *P. pickettii* NCTC 11149. For comparative studies of the total cellular lipids, organisms were grown from spread inocula on nutrient agar (Oxoid CM3) for 48 h at 30 °C (*P. caryophylli*), 24 h at 30 °C (*P. gladioli*), or 24 h at 37 °C (*P. pickettii*). *P. pickettii* was also grown in nutrient broth (Oxoid CM67) for 24 h at 37 °C with aeration at 20 l min⁻¹ and stirring at 300 r.p.m. in a 30-l fermenter (culture volume 20 l). Cells were harvested and washed with water, then freeze-dried.

Extraction and fractionation of lipids. Lipids were extracted with chloroform/methanol (2:1, v/v) and fractionated by stepwise elution from a silica column (Mallinkrodt CC-4) with chloroform containing

Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; OL, ornithine amide lipid.

increasing proportions of methanol (Cox & Wilkinson, 1989). Appropriate fractions were pooled and individual polar lipids were isolated by preparative TLC (solvents A or B). The products were purified by a Folch wash (Duthie & Patton, 1965).

TLC. Separations were carried out with silica gel G or 60 F₂₅₄ (Merck) and the following solvents: A, chloroform/methanol/water (65:25:4, by vol.); B, chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.); C, chloroform/methanol/7 M-ammonia (65:25:4, by vol.); D, chloroform/methanol/acetic acid/water (85:15:10:4, by vol.); E, dichloromethane. Detection reagents used were those listed by Cox & Wilkinson (1989).

Paper chromatography and electrophoresis. Separations were carried out with Whatman no. 1 paper. Solvent F, propan-2-ol/water/18 M-ammonia (7:2:1, by vol.), was used for descending chromatography. Electrophoresis was carried out with pyridine/acetic acid buffers of pH 3.6 or 5.3 (Wilkinson & Caudwell, 1980). Spots were detected by using ninhydrin, the Hanes/Isherwood reagent for phosphates, or the periodate/Schiff reagents for 1,2-diols (Cox & Wilkinson, 1989).

Analysis of polar lipids. In addition to TLC with various solvents and detection reagents, methods used to identify phospholipids included paper chromatography (solvent F) and electrophoresis (pH 3.6 or 5.3) of the water-soluble deacylation products obtained by mild alkaline methanolysis (Kates, 1986). Where appropriate, the presence of ethanolamine was confirmed by acid hydrolysis (2 M-HCl, 2 h, 105 °C) of the deacylation product, followed by paper electrophoresis at pH 5.3. Quantitative data on phospholipid distribution were obtained by ³¹P NMR spectroscopy of the total lipids (London & Feigenson, 1979). Selective *O*-deacylation of ornithine amide lipids was achieved by mild alkaline methanolysis (Kates, 1986). The presence of ornithine in the parent lipid and in the deacylation product (recovered from the upper layer of the biphasic mixture after acidification to pH 6.3) was confirmed by paper electrophoresis (pH 5.3) after total acid hydrolysis (6.1 M-HCl, 4 h, 105 °C).

Analysis of fatty acids. Ester-bound fatty acids were released as methyl esters by alkaline methanolysis of the total or individual lipids, and were quantified by GLC using a fused-silica capillary column of BP1 (25 m) in a Mega 5160 chromatograph (Carlo Erba). Amide-bound acids were released by hydrolysis with 6.1 M-HCl for 4 h at 105 °C, and were esterified either by using ethereal diazomethane or by acid methanolysis (Cox & Wilkinson, 1989). Where necessary, hydroxy and non-hydroxy esters were separated by TLC with solvent E, and their identities were confirmed by MS (Finnigan 1020B). Trifluoroacetylation (Bryn & Jantzen, 1982), monitored by GLC, was also used to diagnose hydroxy esters. Unsaturated esters were isolated by argentation TLC and subjected to catalytic hydrogenation (Cox & Wilkinson, 1989).

Results

Polar lipid composition

The yields of total cellular lipid from agar-grown organisms were as follows: *P. caryophylli*, 10.2%; *P. gladioli*, 9.4%; *P. pickettii*, 8.7%. For the last species, a similar yield (8.3%) was obtained for organisms grown in nutrient broth. TLC of the crude extracts indicated the presence of poly- β -hydroxybutyrate (Palleroni, 1984) and non-lipid contaminants (spots of low mobility on TLC, Fig. 1) in addition to envelope-derived lipids.

Fig. 1. TLC of lipids on silica gel 60 F₂₅₄ with solvent A and detection of amino lipids with ninhydrin. 1, Lipids from *P. caryophylli*; 2, lipids from *P. gladioli*; 3, reference PE; 4, lipids from *P. cepacia* (Cox & Wilkinson, 1989); 5, lipids from *P. pickettii* (agar-grown); 6, lipids from *P. pickettii* (broth-grown).

The extracts from each species contained two major ninhydrin-positive phospholipids (PE1 and PE2, Fig. 1). TLC comparisons with reference PE and the characterized lipids from *P. cepacia* (Cox & Wilkinson, 1989) showed that PE1 and PE2 had the chromatographic properties and reactions of PE containing two non-hydroxy fatty acyl residues or one hydroxy and one non-hydroxy fatty acyl residue, respectively. This inference was confirmed by the formation of *O*-glycerophosphoethanolamine on mild alkaline methanolysis of either lipid, by the release of ethanolamine on acid hydrolysis, and by studies of fatty acid compositions (see below).

Two other ninhydrin-positive lipids (OL1 and OL2, Fig. 1), present in *P. caryophylli* and *P. gladioli*, but in neither culture of *P. pickettii*, did not contain phosphorus and had the chromatographic properties of the ornithine amide lipids produced by *P. cepacia*. The poor resolution of these lipids from PE2 prevented thorough characterization, but the presence of ornithine was confirmed in small samples obtained by preparative TLC for both *P. caryophylli* and *P. gladioli*. The same lyso lipid was apparently produced by mild alkaline methanolysis of both OL1 and OL2, again indicating that the lipids differed only in the nature of their ester-bound acids.

In addition to two forms each of PE and OL, both *P. caryophylli* and *P. gladioli* produced significant amounts of both diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG). By contrast, cultures of *P. pickettii* contained DPG but only small or trace amounts of PG. The distribution of phosphorus between individual phospholipids from agar-grown cells (Table 1) was determined from the ³¹P NMR spectra for the extracts (e.g. Fig. 2).

Fig. 2. ^{31}P NMR spectrum (109.3 MHz) of the phospholipids from *P. gladioli*. Lipids were solubilized by the method of London & Feigenson (1979), and the spectrum was recorded at 20 °C with 85% (w/v) H_3PO_4 as the external reference.

Table 1. Distribution of lipid phosphorus between individual phospholipids

Data for agar-grown cells are expressed as percentages of the total peak area in the ^{31}P NMR spectra (e.g. Fig. 2). tr., Trace.

Organism	Phospholipid			
	PE1	PE2	DPG	PG
<i>P. caryophylli</i>	33	35	13	19
<i>P. gladioli</i>	44	25	20	11
<i>P. pickettii</i>	47	35	18	tr.

Fatty acid composition

The profiles of ester-linked, non-hydroxy fatty acids for total and individual lipids from each species are shown in Tables 2 to 4. The preponderant components were hexadecanoic acid (16:0), a hexadecenoic acid (16:1), an octadecenoic acid (18:1), and the cyclopropanation products from the two monoenoic acids (17:cyc and 19:cyc). Overall, the extent of cyclopropanation was greatest for *P. gladioli* and least for *P. pickettii*, but little can be made of this in the absence of physiological standardization of the cells. Ignoring this aspect, the fatty acid profiles for *P. caryophylli* and *P. gladioli* were very similar; by contrast, the proportions of C_{16} and derived acids in *P. pickettii* were relatively high. No

Table 2. Ester-linked non-hydroxy fatty acid composition of lipids in *P. caryophylli*

Data are expressed as percentages of the total peak area on GLC of the methyl esters prepared by alkaline methanolysis. tr., Trace.

Fatty acid*	Total lipid	PE1	PE2	DPG	PG	OL1
14:0	0.6	tr.	tr.	tr.	0.5	tr.
15:0	0.5	tr.	tr.	tr.	0.6	tr.
16:0	20.2	24.2	24.0	35.1	24.8	35.7
16:1	8.0	6.1	tr.	13.6	9.3	tr.
17:0	0.6	tr.	tr.	tr.	0.5	tr.
17:cyc	17.0	26.9	tr.	15.0	14.6	18.6
18:0	tr.	tr.	tr.	tr.	tr.	tr.
18:1	33.8	27.1	60.7	30.5	29.3	15.4
19:cyc	19.2	15.6	15.2	5.7	20.3	30.2

* Shorthand designation: the number before the colon indicates the total number of carbon atoms, the number after the colon indicates the number of double bonds, and cyc indicates the presence of a cyclopropane ring.

Table 3. Ester-linked non-hydroxy fatty acid composition of lipids in *P. gladioli*

See Table 2 for the method of expressing data and for the fatty acid shorthand used.

Fatty acid	Total lipid	PE1	PE2	DPG	PG	OL1
14:0	0.5	tr.	tr.	tr.	tr.	tr.
15:0	0.4	tr.	tr.	tr.	tr.	tr.
16:0	18.9	29.4	21.7	30.0	33.6	8.7
16:1	3.7	tr.	tr.	4.5	1.8	tr.
17:0	0.6	tr.	tr.	tr.	tr.	tr.
17:cyc	22.0	40.3	4.4	25.9	22.0	11.1
18:0	0.7	tr.	tr.	tr.	tr.	tr.
18:1	22.7	1.0	41.6	14.9	10.0	39.8
19:cyc	30.5	29.2	32.3	24.6	32.6	40.4

Table 4. Ester-linked non-hydroxy fatty acid composition of lipids in *P. pickettii*

See Table 2 for the method of expressing data and for the fatty acid shorthand used.

Fatty acid	Total lipid	PE1	PE2	DPG	PG
14:0	1.2	1.7	2.7	2.0	tr.
15:0	1.1	2.0	3.3	1.7	tr.
16:0	31.8	20.1	36.6	33.3	45.1
16:1	23.9	19.9	6.6	30.3	27.5
17:0	0.8	1.0	1.9	tr.	tr.
17:cyc	12.6	22.3	1.6	6.1	3.8
18:0	tr.	tr.	tr.	tr.	tr.
18:1	27.8	32.2	42.1	26.6	23.6
19:cyc	0.6	0.7	5.2	tr.	tr.

Table 5. Ester-linked hydroxy fatty acid composition of PE2 lipids

Data are expressed as percentages of the total peak area on GLC of the methyl esters from hydroxy acids.

Fatty acid*	<i>P. caryophylli</i>	<i>P. gladioli</i>	<i>P. pickettii</i>
2-OH-16:0	46.5	53.4	25.1
2-OH-16:1	27.4	24.9	44.3
2-OH-17:cyc	tr.	tr.	6.9
2-OH-18:1	12.8	9.1	23.7
2-OH-19:cyc	13.3	12.6	0

* All fatty acids are α -hydroxy (2-OH): shorthand designation as in Table 2.

attempt was made to identify the monoenoic (or cyclopropane) acids rigorously, but argentation TLC of the methyl esters pointed to the *cis* configuration, while GLC retention times matched those for *cis*-16:1(9) and *cis*-18:1(11).

When the non-hydroxy fatty acid profiles (Tables 2 to 4) of individual lipids are compared, the most striking contrast is between PE1 and PE2 for each species. The PE2 member of each pair was markedly depleted in 16:1/17:cyc, but contained a range of 2-hydroxy acids (Table 5). The replacement of a non-hydroxy acid (mainly 16:1 or 17:cyc) by a 2-hydroxy acid clearly accounted for the chromatographic separation of PE2 from PE1. Although there seem to be significant variations in non-hydroxy fatty acid profiles for other phospholipids in the different species, the possibility that these are partly caused by inadvertent fractionation of molecular species during column chromatography or preparative TLC cannot be discounted.

The ornithine amide lipid (OL1) intermediate in TLC mobility between PE1 and PE2 contained ester-linked non-hydroxy acids (Tables 2 and 3). By contrast, the ester-linked acids in OL2 consisted almost entirely of 2-OH-18:1 and 2-OH-19:cyc, together with a small proportion of 2-OH-16:0. The ratio of the two major hydroxy acids in OL2 from *P. caryophylli* was about 1:3; for the lipid from *P. gladioli* the ratio was about 2:1. In both OL1 and OL2, the amide-linked fatty acid was mainly or entirely 3-OH-16:0. Thus, the general structure I can be assigned to both ornithine amide lipids.

Discussion

This study has shown that the unusual phospholipid profile previously reported for *P. solanacearum* (Drigues *et al.*, 1981) and *P. cepacia* (Cox & Wilkinson, 1989) extends to other species (*P. caryophylli*, *P. gladioli* and *P. pickettii*) in *Pseudomonas* homology group II. Although the production of two forms of PE (differentiated by the presence or absence of 2-hydroxy acids) may be a chemotaxonomic feature of the group, the same type of heterogeneity in PE has also been reported for *Streptomyces sioyaensis* (Kawanami *et al.*, 1969), *Nocardia asteroides* (Yano *et al.*, 1970), and probably various other actinomycetes (Pramanik *et al.*, 1990), as well as myxobacteria (Yamanaka *et al.*, 1988), and possibly *Brucella* spp. (Thiele *et al.*, 1971). Thus, such pairs of phospholipids may be more common than is appreciated, and seem to be present in very diverse taxa. Chromatographically distinct fractions of PE have also been described for the alga *Dunaliella bardawil* (Fried *et al.*, 1982) and a psychrophilic *Vibrio* sp. (Okuyama & Sasaki, 1986), but no hydroxy acid was involved in these cases.

In the *Pseudomonas* spp. studied here, the 2-hydroxy acids in PE2 apparently occupy the position on glycerol mainly occupied by 16:1 or 17:cyc in PE1. No attempt was made to identify this position, but in the lipid corresponding to PE2 from *P. solanacearum*, the hydroxy acids [2-OH-16:1(9) and 2-OH-18:1(11)] were in the *sn*-1 position (Drigues *et al.*, 1981). The same distribution apparently applies to PE of *Brucella* spp. (Thiele *et al.*, 1971), but in the phospholipids of *Streptomyces sioyaensis* (Kawanami *et al.*, 1969) and *Nocardia asteroides* (Yano *et al.*, 1970) hydroxy acids were in the *sn*-2 position.

Whereas two forms of PE have been found in all species from homology group II which have been examined, the two ornithine amide lipids were not found in *P. pickettii* (this work and Kawai *et al.*, 1988) and were not reported in a study of the polar lipids of *P. solanacearum* (Drigues *et al.*, 1981). Ornithine amide lipids with the general structure I are very widely distributed in bacteria (Wilkinson, 1988; Lanelle *et al.*, 1990). However, the production of such lipids can depend critically on the cultural conditions used (Wilkinson, 1972; Minnikin & Abdolrahimzadeh, 1974; Wilkinson & Caudwell, 1980; Moule & Wilkinson, 1987; Hara & Cotter, 1987). Thus, further work would be necessary to determine whether the absence of ornithine amide lipids from *P. pickettii* and *P. solanacearum* is a real and distinctive feature of these species, possibly correlated with their association in a separate DNA homology subgroup within RNA homology group II (Palleroni, 1984). The significance of the low level of PG in *P. pickettii* (and no report of this lipid for *P. solanacearum*;

Drigues *et al.*, 1981) is subject to similar uncertainty in view of the dependence of the PG:DPG ratio on the physiological state of the bacteria.

Whereas no studies of the polar lipids of *P. caryophylli*, *P. gladioli*, or *P. pickettii* have been reported previously, other data on total cellular fatty acids are available. Qualitatively, the data for non-hydroxy fatty acids in *P. caryophylli* (Oyaizu & Komagata, 1983), *P. gladioli* (Oyaizu & Komagata, 1983; Dees *et al.*, 1983; Christenson *et al.*, 1989), and *P. pickettii* (Chen & Chen, 1981; Veys *et al.*, 1989) are comparable with those given here. Some disagreement over the identities of monoenoic acids (and the derived cyclopropane acids) may be noted. Although rigorous identification was not attempted in the present study, TLC and GLC results were indicative of *cis*-16:1(9) and *cis*-18:1(11), while oleic acid [*cis*-18:1(9)] and elaidic acid [*trans*-18:1(9)] were clearly eliminated as possibilities. These inferences were essentially in accord with previous data for *P. gladioli* (Dees *et al.*, 1983; Christenson *et al.*, 1989). On the other hand, the 18:1 component in *P. pickettii* was indicated by Veys *et al.* (1989) to be a mixture of *cis*- and *trans*-18:1(9).

In previous studies, no attempt was made to determine the origins of particular fatty acids, nor to compare the fatty acid profiles of individual lipids. Thus, the hydroxy acids detected in analyses of total cellular fatty acids could have arisen from PE₂, the ornithine amide lipids, or lipopolysaccharide. It seems likely that most of the α -hydroxy acids found for *P. gladioli* (Dees *et al.*, 1983; Christenson *et al.*, 1989), similar to those listed in Table 5, were derived from PE₂ and OL₂, while some of the 3-OH-16:0 found for this species and for *P. caryophylli* was probably derived from ornithine amide lipids and the remainder from lipopolysaccharide.

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