Lipid composition and taxonomy of [*Pseudomonas*] *echinoides*: transfer to the genus *Sphingomonas*

Nicola J. Rowe, Jennifer Tunstall, Lesley Galbraith
and Stephen G. Wilkinson

Author for correspondence: Stephen G. Wilkinson. Tel: +44 1482 651152. Fax: +44 1482 466410.

Department of Chemistry,
University of Hull,
Hull HU6 7RX, UK

Lipid components of [*Pseudomonas*] *echinoides* NCIMB 9420 have been studied as an aid to taxonomic relocation of the organism. Non-polar lipids include the carotenoid nostoxanthin and the ubiquinone Q-10. The major fatty acids are *cis*-vaccenic acid [18:1(11c)], hexadecanoic acid (16:0) and 2-hydroxy-tetradecanoic acid (2-OH-14:0), but 11-methyloctadec-11-enoic acid [11-Me-18:1(11)] is a significant minor component. The preponderant phospholipids are phosphatidylethanolamine and phosphatidylglycerol; minor lipids include bis(phosphatidyl)glycerol and an unidentified aminophospholipid. Several glycolipids are present, the major one being a glucuronosylceramide derived from sphinganine with amide-bound 2-OH-14:0. The lipid profile supports a proposal to reclassify the organism as *Sphingomonas echinoides*.

Keywords: *Pseudomonas echinoides*, *Sphingomonas*, lipids

INTRODUCTION

[*Pseudomonas*] *echinoides*, a little-studied pseudomonad, was first isolated as a laboratory contaminant (Heumann, 1960). The organism is a yellow rod with a single polar flagellum and only the one strain seems to have been deposited in culture collections (ATCC 14820/NCIMB 9420/DSM 1805/ICPB 2835). The organism was left in Section V of *Pseudomonas* (miscellaneous species) by Palleroni (1984). However, subsequent studies of DNA–rRNA hybridization showed that [*P.*] *echinoides* did not belong to the genus *Pseudomonas* as now defined, but it was placed at the base of rRNA superfamily IV (De Vos *et al.*, 1989). Other false pseudomonads in the superfamily (De Vos *et al.*, 1989; Van Bruggen *et al.*, 1993) include [*Pseudomonas*] *paucimobilis*, which belongs to the α -4 subclass of the *Proteobacteria* (Takeuchi *et al.*, 1994) and is now the type species of the genus *Sphingomonas* (Yabuuchi *et al.*, 1990, 1999). A relationship between [*P.*] *echinoides* and *Sphingomonas paucimobilis* has also been suggested (Owen & Jackman, 1982; De Vos *et al.*, 1989) from similarities in DNA base composition, partial DNA–

DNA hybridization and the presence in both species of the carotenoid nostoxanthin (Jenkins *et al.*, 1979).

Lipid profiles have found many applications in bacterial chemotaxonomy, including the circumscription of *Sphingomonas* spp. Distinctive features of these organisms include the absence of 3-hydroxy acids (markers for lipopolysaccharide *inter alia*), but the presence of 2-hydroxytetradecanoic (2-OH-14:0) and octadecenoic (18:1) acids as major components, together with a range of glycosphingolipids (Yamamoto *et al.*, 1978; Yabuuchi *et al.*, 1979, 1990, 1999; Kawahara *et al.*, 1991; Mizuno *et al.*, 1992; Takeuchi *et al.*, 1993, 1994, 1995; Kämpfer *et al.*, 1997). Characterization of the lipids and fatty acids from [*P.*] *echinoides*, the object of this study, could therefore clarify the classification of this organism. When this study was almost complete, the results of a detailed chemotaxonomic analysis of [*P.*] *echinoides* (including lipid data) and a formal proposal to reclassify the species as *S. echinoides* were published (Denner *et al.*, 1999). The results of our own study support and extend these observations.

METHODS

Organism and growth conditions. Two batches of [*P.*] *echinoides* strain NCIMB 9420 were grown on nutrient agar (Oxoid CM3) for 48 h at 37 °C (A) or for 72 h at 30 °C (B). Bottles (100), each containing 50 ml medium, were inoculated

Abbreviations: DPG, bis(phosphatidyl)glycerol; HPAEC, high-pH anion-exchange chromatography; HVE, high-voltage electrophoresis; L-PE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; TMS, trimethylsilyl.

with a suspension of cells washed from starter slopes with sterile nutrient broth (Oxoid CM67). The yields of freeze-dried, washed cells were 0.84 g (A) and 3.0 g (B). A further batch of cells (C, yield not determined) was grown on enriched cytophaga solid medium 98 (NCIMB) for 48 h at 30 °C. *S. paucimobilis* strain NCTC 11030 was grown on medium 98 for 24 h at 30 °C as a source of reference lipids.

Extraction and fractionation of lipids. Lipids were extracted by stirring dry cells with chloroform/methanol (2:1, v/v) for 2 h at room temperature. To avoid possible losses of polar glycolipids, no attempt was made to remove non-lipid contaminants from the extracts. Lipid solutions were stored in the dark at -20 °C. Fractions soluble in acetone and hexane were used for the examination of carotenoids and isoprenoid quinones, respectively.

Chromatographic and electrophoretic methods. In general, TLC separations were carried out on silica gel 60F₂₅₄ (Merck) with the following solvent systems: A, chloroform/methanol/acetic acid (65:25:10, by vol.); B, chloroform/methanol/acetic acid/water (100:20:12:5, by vol.); C, chloroform/methanol/water (65:25:4, by vol.); D, chloroform/methanol/acetic acid/water (80:12:15:4, by vol.); E, ethyl acetate/pyridine/water (13:5:4, by vol.); F, light petroleum (b.p. 60–80 °C)/acetone (19:1, v/v); G, dichloromethane. Aminolipids were detected with ninhydrin, phospholipids with Dittmer–Lester reagent, glycolipids with α -naphthol/sulphuric acid and lipids containing 1,2-diol or 1-hydroxy-2-amino groups with periodate/Schiff reagents (Christie, 1982). Other methods used to detect lipids were exposure to UV light (for isoprenoid quinones) or iodine vapour, and spraying with ethanolic 2',7'-dichlorofluorescein. Reverse-phase TLC of isoprenoid quinones was carried out on RP-18F₂₅₄ plates (Merck) with solvent H (acetone/acetonitrile, 4:1, v/v). HPLC using a C₁₈ column (25 cm $\times$ 4.6 mm) eluted with solvent I (methanol/propan-2-ol, 3:1, v/v) and monitored at 270 nm, was also used to identify the major quinone. High-pH anion-exchange chromatography (HPAEC) of monosaccharides utilized a CarboPac PA100 column in a Dionex DX-300 system. Neutral sugars were eluted with 0.016 M NaOH, amino sugars with 0.25 M NaOH and hexuronic acids with 0.015 M NaOH (20 min) followed by a linear gradient (20 min) to 0.15 M sodium acetate/0.1 M NaOH (10 min).

GLC separations were carried out with either a Carlo Erba Mega 5160 or a Perkin-Elmer Autosystem XL chromatograph, fitted with fused-silica capillary columns (25 m) of BP1 (SGE). Finnigan MAT 1020B and GCQ instruments were used for GLC-MS. Paper chromatography and high-voltage electrophoresis (HVE) were carried out with Whatman no. 1 paper and the following solvents and buffers: E; J, ethyl acetate/pyridine/water/acetic acid (5:5:3:1, by vol.); K, pyridine/acetic acid/water (5:2:43, by vol., pH 5.3); L, pyridine/acetic acid/water (1:10:89, by vol., adjusted to pH 2.7 with formic acid). Detection reagents used were ninhydrin, aniline hydroxoxalate, alkaline AgNO₃ and the reagent of Hanes & Isherwood (1949) for phosphate esters.

Studies of polar lipids. Phospholipids were initially characterized by TLC (solvent systems A–D) and by HVE (buffer K) at pH 5.3 of the water-soluble deacylation products. Confirmation of identity and quantitative data were provided by ³¹P NMR spectroscopy (London & Feigenson, 1979), using a JEOL JNM-LA400 spectrometer. Sphingoid bases were released by acid methanolysis (Yano *et al.*, 1982) of total lipids at 60 °C for 20 h. After extraction of fatty methyl esters with hexane, the hydrolysate was basified (KOH) and re-

extracted with hexane/diethyl ether (1:1, v/v). The products were examined by TLC (solvent C, detection with ninhydrin and periodate/Schiff reagents) and, as the trimethylsilyl (TMS) derivatives (Yano *et al.*, 1982), by GLC-MS. Analysis for lipid carbohydrate (as glucose) was carried out by a modified phenol/sulphuric acid assay (Kushwaha & Kates, 1981), and the method of Blumenkrantz & Asboe-Hansen (1975) was applied for the detection of hexuronic acids. Monosaccharide components of lipids were released by hydrolysis with 2 M trifluoroacetic acid at 98 °C for 16 h (Yu Ip *et al.*, 1992). Neutral sugars were identified by paper chromatography and TLC (both solvent E), HPAEC and GLC of the alditol acetates. The D configuration of glucose was assigned by using D-glucose oxidase (EC 1.1.3.4). Basic and acidic sugars were identified by paper chromatography (solvent J), HVE (buffers K and L) and HPAEC.

Studies of fatty acids. Fatty acids were released as methyl esters by mild alkaline (Barnes *et al.*, 1989) or acid methanolysis (Yano *et al.*, 1982), or by acid hydrolysis (6.1 M HCl, 100 °C, 4 h) of the lipids followed by reaction with diazomethane. The products were analysed by GLC and by GLC-MS. Saturated and monoenoic esters were separated by argentation TLC (solvent G) and unsaturation was confirmed by hydrogenation. For the location of double bonds, the methyl esters were hydrolysed (Aveland & Horrocks, 1983), converted into 3-picolinyl esters (Wait & Hudson, 1985) and these were also examined by GLC-MS.

Studies of non-polar lipids. Yellow pigments in extracts from [*P.*] *echinoides* were examined by TLC (solvents A and C) and the visible spectrum of a solution in acetone was recorded with a Philips PU8720 scanning spectrophotometer. The presence of isoprenoid quinones was monitored by TLC (solvent F), reverse-phase TLC and HPLC as described above: material isolated by HPLC was examined by MS.

RESULTS

Because [*P.*] *echinoides* grew poorly in all complex liquid media tried, cells for lipid studies were obtained by surface culture. Unpurified lipid extracts constituted 11–12% of the cell dry weight (batches A and B) and were bright yellow. Two yellow spots (one major, one minor) with high *R_F* values, greater than those of the most mobile phospholipid, bis(phosphatidyl)glycerol (DPG), were detected by TLC. The visible spectrum obtained for the total acetone-soluble material from batch A, and also for the major, isolated pigment (*R_{DPG}* 1.17, solvent A), matched that reported for bacterial nostoxanthin (Yabuuchi *et al.*, 1979; Jenkins *et al.*, 1979), consistent with the reported presence of this carotenoid in *S. paucimobilis* and [*P.*] *echinoides*. Analysis of hexane-soluble products in lipid batch B showed the presence of ubiquinone Q-10, also a distinctive feature of *Sphingomonas* spp. (Yabuuchi *et al.*, 1999).

Fatty acid profiles for all three batches of lipid were qualitatively similar: the illustrative data shown (Table 1) were determined for lipid batch B. The profile obtained after mild alkaline methanolysis was dominated by the methyl esters of just two fatty acids, hexadecanoic acid (16:0) and a *cis*-octadecenoic acid [18:1(*c*)]. The latter, preponderant component was identified as 18:1(11*c*) (*cis*-vaccenic acid) by GLC-MS of

Table 1. Fatty acid composition of the total lipids of [*P.*] *echinoides*

Data are expressed as percentages of the total peak area on GLC of the methyl esters obtained by mild alkaline methanolysis or acid methanolysis of lipid batch B. TR, Trace.

Fatty acid*	Mild alkaline methanolysis	Acid methanolysis
14:0	TR	TR
15:0	0.5	TR
2-OH-14:0	0	30.6
16:1	2.2	2.2
16:0	23.7	23.0
17:1	1.4	TR
17:0	0.7	TR
18:1(11 <i>c</i>)	62.2	40.1
18:1	TR	TR
18:0	2.6	TR
11-Me-18:1(11)	6.6	3.5

* Shorthand designation. The number before the colon indicates the total number of carbon atoms, the number after the colon the number of double bonds, the number in parentheses the position of the double bond and *c* the *cis* isomer.

the 3-picolinyl ester (Harvey, 1982; Christie *et al.*, 1987). Among the minor components was an unsaturated acid, the methyl ester of which had a GLC retention time a little greater than that of methyl octadecanoate but migrated on argentation TLC just ahead of reference *cis*-monoenoate esters. MS indicated that this was a C₁₉ monoenoic acid (molecular ion with *m/z* 310). These properties were consistent with a methyl-branched 18:1 acid and MS of the 3-picolinyl ester revealed a spectrum identical to that of the 11-methyloctadec-11-enoic ester from *Brevundimonas* (*Pseudomonas*) *vesicularis* (Wilkinson & Galbraith, 1979; Barnes *et al.*, 1989). When fatty esters of [*P.*] *echinoides* were prepared by acid methanolysis of the lipids, or by acid hydrolysis followed by treatment of the free acids with diazomethane, a third major component was detected and identified as the ester of 2-hydroxytetradecanoic acid (Table 1). Failure to release this component by mild alkaline methanolysis pointed to an amide linkage.

The major phospholipids in extracts from [*P.*] *echinoides* were identified by TLC and by HVE of the deacylation products as phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), which accounted for 41 and 32%, respectively, of the lipid phosphorus in batch C (similar proportions were found for batch B). Also apparently present was DPG (9% of the lipid phosphorus), although small differences in mobility between the bacterial product and reference lipid were consistently observed by both TLC (e.g. Fig. 1) and HVE. In addition, extracts contained a little P_i [detected by ³¹P NMR spectroscopy of the intact lipids (Fig. 2, signal at 2.66 p.p.m.) and by HVE of the deacylation products] and an unidentified aminophospholipid (15% of the

Fig. 1. TLC of the phospholipids of [*P.*] *echinoides* in solvents A and B. Std, reference lipids; Test, lipid batch C; X, unidentified aminophospholipid.

lipid phosphorus). On TLC, the latter had a mobility intermediate between PE and lysophosphatidylethanolamine (L-PE), as illustrated in Fig. 1, and was assumed to give the unattributed signal at 0.64 p.p.m. in the ³¹P NMR spectrum (Fig. 2). This unidentified lipid was absent from *S. paucimobilis*.

The presence in the lipids of [*P.*] *echinoides* of amide-bound 2-OH-14:0 and the relationship with *Sphingomonas* spp., prompted a search for (glyco)sphingolipids. After acid methanolysis of the total lipids, a basic, lipophilic product was obtained. On TLC, a spot with the mobility of sphingosine was detected with ninhydrin and periodate/Schiff reagents. The product gave a TMS derivative with a GLC retention time close to that of the sphingosine and GLC-MS showed peaks characteristic

Fig. 2. ^{31}P NMR spectrum of the phospholipids of [*P.*] *echinoides*. Lipids (batch C) were solubilized by the method of London & Feigenson (1979) and the spectrum was recorded at 162 MHz and ambient temperature with the signal for PE set at 0.00 p.p.m. The signal at 2.66 p.p.m. could be attributed to P_i and that at 0.64 p.p.m. was provisionally assigned to the unidentified aminophospholipid X (see Fig. 1).

of the TMS derivatives of sphingoid bases, including the base peak with m/z 132 (Yano *et al.*, 1982). Fragment ions with m/z 430 [M-15], 342 [M-103] and 313 [M-132] showed that the sphingoid base was a saturated C_{18} dihydroxyamine, probably 2-aminooctadecane-1,3-diol (dihydrosphingosine or sphinganine).

Colorimetric tests indicated the presence in the lipids of carbohydrate, estimated at 6% in batch B (expressed as glucose), including a hexuronic acid. Extensive analyses for monosaccharide components showed a difference between the two lipid batches examined (B and C). Both contained glucuronic acid and glucose, but batch B also contained galactose (the major hexose), glucosamine and probably minor amounts of rhamnose and mannose. When α -naphthol/sulphuric acid was used to visualize the parent lipids on TLC, several positive reactions were observed. The most intense spot ($R_{\text{PE}} \sim 0.4$ in solvent A) matched the major glycolipid in *S. paucimobilis* and was unaffected by mild alkaline methanolysis, consistent with its identification as a glucuronosylceramide (SGL-1; Yabuuchi *et al.*, 1999). Three less intense bands of lower R_{F} values could correspond to glycosphingolipids produced by further glycosylation and/or non-lipid contaminants. Several other glycolipid bands, with TLC mobilities greater

than PE, were detected and could include a mono-glycosyldiacylglycerol. Further studies on isolated components are necessary for structural characterization of the unidentified lipids described for [*P.*] *echinoides*.

DISCUSSION

This survey of [*P.*] *echinoides* has revealed or confirmed a distinctive lipid profile for this species. In addition to three common phospholipids (PE, PG and DPG), an unidentified aminophospholipid is a significant component, and one not found in *S. paucimobilis*. Both organisms also produce glycosphingolipids and the parent ceramide in [*P.*] *echinoides* apparently has 2-OH-14:0 amide-linked to the saturated C_{18} base sphinganine. In the glycosphingolipids of *Sphingomonas* spp., the same ceramide is accompanied by others containing related sphingoid bases (Yamamoto *et al.*, 1978; Kawahara *et al.*, 1991; Mizuno *et al.*, 1992; Takeuchi *et al.*, 1993, 1994, 1995; Yabuuchi *et al.*, 1999), which might have eluded detection in the present study. Substitution of the ceramides by α -D-glucuronic acid produces the simplest glycosphingolipid (SGL-1), further stepwise glycosylation of which leads to a tetrasaccharide derivative (SGL-4) of the following structure: α -D-Manp-(1 $\rightarrow$ 2)- α -D-Galp-(1 $\rightarrow$ 6)- α -D-GlcpN-(1 $\rightarrow$ 4)- α -D-GlcpA-(1 $\rightarrow$ 1)-ceramide (Kawahara *et al.*, 1991; Mizuno *et al.*, 1992). The preliminary data on the monosaccharide composition of lipids from [*P.*] *echinoides* suggest a similar extension of SGL-1 in this organism: the failure to detect glucosamine in lipid batch C could be a consequence of the resistance to acid-catalysed cleavage of the glycosidic bonds formed by hexosamines and hexuronic acids.

Overall, the results of this investigation are in accord with other studies of the carotenoid (Jenkins *et al.*, 1979) and other lipid components (Denner *et al.*, 1999) of [*P.*] *echinoides*. In the latter study, the major isoprenoid quinone was identified as Q-10 and the principal fatty acids as 2-OH-14:0, 16:0 and 18:1. Based on the results of two-dimensional (2D) TLC, the major phospholipids were reported as PE and PG; other phospholipids found included an unidentified aminophospholipid and a spot corresponding to SGL-1 was visualized. No further characterization of the polar lipids was apparently attempted, no information on sphingoid base nor monosaccharide composition was presented and the 11-Me-18:1(11) fatty acid was not described. On the other hand, the use of 2D-TLC provided enhanced resolution of the polar lipids and detection of several other unidentified (glyco/phospho)lipids. Both the results of Denner *et al.* (1999) and the present study support the transfer of [*P.*] *echinoides* to the genus *Sphingomonas*. Within the genus there is some diversity of lipid composition, e.g. [*P.*] *echinoides* lacks the methylated derivatives of PE (including phosphatidylcholine) found in other *Sphingomonas* spp. (Kämpfer *et al.*, 1997; Denner *et al.*, 1999) and there can be significant variations in fatty acid composition. The preponderant 18:1 acid in several species has been listed as 18:1(9) by

Yabuuchi *et al.* (1999) in contrast to 18:1(11c) found here in [*P.*] *echinoides*. In other studies, the relative abundance of individual members of the dominant envelope of GLC peaks [18:1(6t), 18:1(9t), 18:1(11c)] was not reported (Kämpfer *et al.*, 1997; Denner *et al.*, 1999). The fatty acid provisionally identified here as 11-Me-18:1(11) has not been found in other *Sphingomonas* spp. but, interestingly, it (or another methyl-branched monoenoic acid) has been described for the lipids from other organisms (*Rhizobium*, *Bradyrhizobium*, *Brevundimonas*, *Caulobacter* and *Hyphomonas* spp.) belonging to the α -subclass of the *Proteobacteria* (Gerson *et al.*, 1975; Gerson & Patel, 1975; MacKenzie *et al.*, 1979; Andreev *et al.*, 1986; Barnes *et al.*, 1989; Abraham *et al.*, 1997, 1999).

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