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LIPOPOLYSACCHARIDES FROM THREE PHYTOPATHOGENIC PSEUDOMONADS

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Key Word Index—*Pseudomonas amygdali*; *P. syringae* pv. *ciccaronei*; *P. syringae* subsp. *savastanoi*; phytopathogenic bacteria; lipopolysaccharides; *O*-chain; fatty acids; chemotaxonomy.

Abstract—Analysis of the polysaccharide and lipid moieties of the lipopolysaccharides (LPSs) of the phytopathogenic bacteria, *Pseudomonas amygdali* and *P. syringae* pv. *ciccaronei* has demonstrated that for both bacteria the *O*-chain consists of a tetrasaccharide repeating unit of three α-L-Rha*p* and one terminal non-reducing α-D-Fuc*p*3NAc. Two of the rhamnosyl residues are 3-linked, the third one 2,3-linked. This structure had been previously found for the *O*-chains of three phytopathogenic strains of *P. syringae* subsp. *savastanoi*, but this is the first report on its occurrence in *P. amygdali* and *P. syringae* pv. *ciccaronei*. The results of the LPS lipid residue analysis made it possible to make some chemotaxonomic considerations and therefore classify *P. amygdali* as a chemotype, which is different from that of the other two bacteria examined. © 1997 Elsevier Science Ltd

INTRODUCTION

Pseudomonas syringae subsp. *savastanoi* (= *P. savastanoi*), *P. amygdali* and *P. syringae* pv. *ciccaronei* (= *P. ciccaronei*) are responsible for olive and oleander knot disease [1], hyperplastic bacterial canker on almond plants [2] and bacterial leaf spots on carrob plants [3], respectively.

Pseudomonas savastanoi and *P. amygdali* share certain phytopathological traits. In particular, both bacteria produce indolacetic acid and cytokinins which are necessary for symptoms to develop on their respective host plants [4, 5]. As expected, these two bacteria can be separated into distinct groups on the basis of numerical [6] and cellular fatty acid analyses [7]. The present taxonomic position of *P. ciccaronei* is uncertain in relation to the other organisms, but it is clear that they differ significantly in terms of the type of disease caused and mechanism of symptom induction.

Recently, the structure of the *O*-chain of three *P. savastanoi* strains was described [8]. In each strain, it consisted of a tetrasaccharide repeating unit of three α-L-Rha*p* and one terminal non-reducing α-D-Fuc*p*3NAc. Two rhamnosyl residues are 3-linked and the third one is 2,3-linked. This study was undertaken in order to gain taxonomic information from analyses of lipopolysaccharides (LPSs) and also to verify whether these molecules were involved in host recognition by *P. savastanoi* strains. In the present paper, we compare the *O*-chain chemical structure and the fatty acid composition of the LPSs of *P. savastanoi* with those of *P. amygdali* and *P. ciccaronei* and show that the three bacteria have the same *O*-chain chemical structure, while the fatty acids differ in their quantitative composition.

RESULTS AND DISCUSSION

Two polysaccharide components from the crude cell material of *P. amygdali* were obtained by gel-chromatography. The less retained component was identified as levan, a fructose homopolysaccharide mostly consisting of (2 → 6) linkages [9] on the basis of its ^{13}C NMR spectra which showed signals at δ 105.2 (quaternary carbon), 81.3, 77.3 and 76.2 (methine carbons), and 64.4 and 60.9 (methylene carbons) [9]. Thispolysaccharide is often a component of the exopolysaccharide fraction of bacteria [10] and, in our

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case, it occurs as a capsular polysaccharide, which is bound to the external surface of the bacterium. The more retained component was constituted by LPS. In the case of *P. ciccaronei*, only one peak, due to the LPS, was obtained by gel-chromatography of the cell material extract.

The ^1H and ^{13}C NMR spectra of the *O*-chains, obtained by acid hydrolysis of LPSs for both *P. amygdali* and *P. ciccaronei*, showed four anomeric signals of similar intensity at δ 4.85 (*br s*) 4.88 (*d* 3.9 Hz), 4.95 (*br s*) and 5.04 (*br s*) and at δ 96.3, 101.3, 102.1 and 102.7, respectively. These data suggested the same *O*-chain repeating unit **1** for both bacteria, as was previously found for the LPSs of *P. savastanoi* strains [8].

$$\rightarrow 3)\text{-}\alpha\text{-L-Rha}p(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p(1\rightarrow$$
$$\begin{array}{c} 3 \\ \uparrow \\ 1 \\ \alpha\text{-D-Fuc}p3\text{NAc} \end{array}$$
1

In order to confirm this, we submitted the *O*-chains of LPSs of both bacteria to classical chemical analyses for polysaccharides. Acid hydrolysis of *O*-chains followed by the GC analysis of the monosaccharides as alditol acetates only revealed the presence of rhamnose and 3-deoxy-3-acetoamidofucose in a *ca* 3:1 ratio. A similar indication was obtained from GC analysis of the trimethyl silyl derivatives of methyl glycosides. Methylation of the *O*-chains indicated the presence of 3-linked rhamnopyranose, 2,3-linked rhamnopyranose and terminal 3-deoxy-3-acetoamidofucopyranose residues, confirming the type of interglycosidic linkages shown in formula **1**.

As far as the configuration of anomeric centres is concerned, the sequence of monosaccharides and their absolute configurations were deduced by the identity of ^1H and ^{13}C NMR data of the *O*-chains with those already described by us for the *O*-chains of LPSs from *P. savastanoi* strains [8]. The data in Table 1 indicated size similarity in the *O*-chains for all of the bacteria, except for PVFi5, for which it was not determined.

This is the first report of the identification of the LPS *O*-chain chemical structure of *P. amygdali* and *P. ciccaronei*. The occurrence of a similar chemical structure for the *O*-chain of LPSs of the above pseudomonads, which are pathogenic on different species, suggests no relationship between *O*-chain structure and host-specificity. In this regard, it is worth noting that the same *O*-chain structure has been found for *P. siringae* pv. *tomato*, a bacterium pathogen for tomato [11].

Table 1. M_r and $[\alpha]_D^{25}$ data of the *O*-chain of lipopolysaccharides of *Pseudomonas* species *P. savastanoi*, *P. amygdali* and *P. ciccaronei*

Species	M_r	$[\alpha]_D^{25}$
P. savastanoi ITM317*	53 000	+6.5
P. savastanoi ITM419*	53 000	not determined
P. amygdali	38 000	+17
P. ciccaronei	67 000	+10

* From ref. [8].

In order to extend the comparison between the chemical structure of the LPSs, we investigated the lipid moieties of the LPSs of the three different strains of *P. savastanoi* (ITM317, ITM519 and PVFi5), *P. amygdali* and *P. ciccaronei*. The data in Table 2 show that the qualitative fatty acid composition was the same for all of the bacteria investigated, the only difference being in the quantitative composition of *P. ciccaronei* and *P. amygdali* with respect to the very similar lipid composition of *P. savastanoi* strains. In particular, in *P. ciccaronei* LPS the 3-OH 10:0 was present in a larger amount, whereas in *P. amygdali* LPS the 2-OH 12:0 only appeared in traces. However, these differences were not so important to ascribe to them the host-specificity for these bacteria. It has also been reported for some *P. savastanoi* bacteria that no relationship exists between the total cellular lipid composition and virulence [12].

From the chemotaxonomic point of view, pseudomonads have been classified on the basis of the serogroups and of the chemotypes concerning the *O*-chain, the core and the lipid composition of LPSs [13]. On the basis of the *O*-chain chemotype, all of the above bacteria can be classified as I chemotype, the same as *P. siringae* pv. *tabaci* [13]. However, they can be differentiated according to their lipid content. *Pseudomonas amygdali* can be classified as chemotype III, the same as *P. savastanoi* pv. *tabaci* because its LPS only contains traces of 2-OH 12:0, while all of the other bacteria would belong to chemotype I. A more detailed classification of plant-pathogenic pseudomonads, mainly based on the content of fatty acids [7], also puts *P. amygdali* in a particular group, but this classification is based on the entire content of the cellular fatty acids.

Finally, it is worth noting that the three *P. savastanoi* strains can be differentiated on the basis of their plasmid profiles [14, 15] and are included in three different groups by DNA fingerprinting [16], thus suggesting that LPS composition cannot be used to differentiate bacteria below the species level.

EXPERIMENTAL

General. ^1H and ^{13}C NMR were obtained at 400 and 100 MHz, respectively, with a spectrometer equipped with a dual probe, in the Fourier transform mode at 50°. ^{13}C and ^1H chemical shifts were measured in D_2O solns using 1,4-dioxane (67.4 ppm) and TSP (sodium 3-trimethylsilypropionate-2,2,3,3-d_4), respectively, as int. standards. Total carbohydrates were determined by the PhOH–H_2SO_4 test [17]. UV absorbance was determined in H_2O solns. Alditol acetates were analysed by GC-MS on an SP-2330 capillary column

Table 2. Molecular ratios of fatty acid components of lipopolysaccharides of *Pseudomonas* species

Fatty acids	*P. savastanoi* ITM317	*P. savastanoi* ITM519	*P. savastanoi* PVFi5	*P. amygdali*	*P. ciccaronei*
3-OH 10:0	0.97	0.85	0.94	0.70	1.70
12:0	1.00	1.00	1.00	1.00	1.00
2-OH 12:0	0.93	1.10	0.84	0.07	0.90
3-OH 12:0	0.34	0.37	0.24	0.26	0.34
16:0	0.02	0.02	0.03	0.01	0.02

(Supelco, 30 m × 0.25 mm i.d., flow rate 0.8 ml min^{-1}) at 240° for 40 min, using He as carrier gas. Partially methylated alditol acetates were analysed by GC-MS, on the above column, with the temp. programme: 80° for 2 min, 80–170° at 30° min^{-1}, 170–240° at 4° min^{-1} for 30 min. Methylation was performed according to Hakomori [18] with KCH_2SOCH_3, MeI in DMSO. Methylated products were recovered by adsorption on C_{18} Sep-Pak. GC of the partially methylated alditol acetates was carried out on an identical column to that used for GC-MS. Optical rotations were determined in H_2O solns.

Bacterial strains. Three strains of *P. savastanoi* were used in this study: oleander-strain ITM519, olive-strain ITM317 (from the collection of the Istituto Tossine e Micotossine da Parassiti Vegetali del CNR, Bari, Italy) and the atypical olive-strain PVFi5 (from the collection of the Istituto di Patologia e Zoologia Forestale ed Agraria, Università di Firenze, Italy) which does not give rise to a fluorescent pigment and produced levan. The two strains of *P. amygdali* NCPPB2610 and *P. ciccaronei* NCPPB2355 were obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, England.

Media and growth conditions. Liquid cultures of *P. savastanoi* were obtained by growing the strains (ITM519, ITM317 and PVFi5) in a nutrient glycerol broth (NBG) and/or King's medium B (KB) with the same conditions as previously reported [8]. *P. amygdali* and *P. ciccaronei* were grown on Wolley's medium supplemented with 1.5% peptone (Difco) as previously reported [19]. Cells were harvested by centrifugation and filtration, and then lyophilized.

Preparation of cellular lipopolysaccharides. In all cases, the dry cells obtained from culture filtrates were extracted with PhOH [20]. The polysaccharide aq. phase was dialysed, lyophilized and the residue treated to eliminate nucleic acid components. Crude LPSs were then obtained by pptn with cold EtOH [8]. As far as ITM317, ITM519 and PVFi5 strains of *P. savastanoi* strains are concerned, the yield in LPS was 110, 78 and 17 mg when the strains are grown on NBG (2.40, 1.04 and 0.98 g of dry cells per 3.2, 2.4 and 3.2 l of culture filtrates, respectively) and 96 mg when the strain ITM519 was grown in KB (2.16 g of dry cells per 3.1 l of culture filtrates); for *P. amygdali* (4.3 g dry cells per 2.7 l of culture filtrates) and *P. ciccaronei* (2.5 g dry cells per 2.3 l of culture filtrates) the yields were 240 and 201 mg of 'crude' LPS, respectively.

Purification of LPS. Crude LPS samples from both *P. amygdali* (240 mg) and *P. ciccaronei* (185 mg) were purified separately by chromatography on Bio-Gel A15m (Bio-Rad) [8], using as eluant a soln of 300 mM Et_3N (TEA) neutralized to pH 7 with HCl. The samples were dissolved in a soln of 300 mM Et_3N and 10 mM EDTA, adjusted to pH 7 with HCl prior to application to the column. Frs were collected and monitored for carbohydrate and UV absorbance at 280 and 260 nm in order to verify the presence of protein and nucleic acid, respectively. Frs containing carbohydrate were combined and dialysed against distilled H_2O for one week. In the case of *P. amygdali*, two peaks were collected, the less retained (40 mg) constituted by levan, the other by purified LPS (141 mg), while for *P. ciccaronei* only one carbohydrate peak was collected and after lyophilization gave 48 mg of purified LPS.

Isolation of O*-chain from LPS.* The purified LPS of each bacterium was hydrolysed, in 1 M HOAc in a sealed tube for 3 hr at 110° [8]. After cooling, lipids were removed by centrifugation. The supernatant was neutralised and lyophilised, dissolved in H_2O and applied to Bio-Gel P-10 (2.5 × 47 cm). The sample was eluted by H_2O at a flow rate of 85.5 ml hr^{-1}. Frs were analysed for carbohydrate. The high M_r carbohydrate frs (*O*-chain) from *P. amygdali* were combined and lyophilised (75 mg) $[\alpha]_D^{25}$ +17.0 (*c* 0.2, H_2O) and from *P. ciccaronei* (24 mg) $[\alpha]_D^{25}$ +10 (*c* 0.08, H_2O). The M_rs of the *O*-chains were estimated by gel-filtration on Bio-Gel A 0.5 m for *P. amygdali* (38 000), and on a Bio-Gel P-100 column for *P. ciccaronei* (67 000). In both cases, the columns were calibrated with dextran standards and 50 mM NaOAc, pH 5.2, solns were used as eluants.

Hydrolysis and methylation of O*-chains.* A sample (1 mg) of the *P. amygdali O*-chain was suspended in 200 μl of 2M H_2SO_4. HOAc (1.8 ml) was added and the soln heated at 100° for 9 hr in a sealed tube. After removal of HOAc, by repeated co-distillations with H_2O *in vacuo*, H_2SO_4 was neutralised by the addition of H_2O (1 ml) and $BaCO_3$ (120 mg). The slurry was centrifuged. $NaBH_4$ (5 mg) was added to the supernatant and the mixt. left for 1 hr at room temp. After neutralization with HOAc and H_2O evapn, the sample was acetylated with pyridine–Ac_2O (1:2, 20 min at

120°). After evapn *in vacuo*, alditol acetates were extracted with CH_2Cl_2 from the residue and analysed by GC. A sample of *O*-chain (1 mg) was methylated (see *General*) and then treated as described above. GC-MS and GLC analysis gave 2,4-di-*O*-methyl-rhamnose, 4-*O*-methyl-rhamnose and 3,6-dideoxy-2,4-di-*O*-methyl-3-*N*-methylacetamido-galactose. The same procedure was used for acid hydrolysis and methylation of the corresponding samples of *P. ciccaronei O*-chain.

Methanolysis of LPSs. LPS (2 mg) was dissolved in 1M HCl–MeOH (1 ml) and kept at 80° for 20 hr. After neutralisation with Ag_2CO_3 and centrifugation, the supernatant liquor was treated with hexane–MeOH (1:1). The hexane phase containing the esterified lipids was analysed by GC on SPB-1 with the temp. programme: 160° for 3 mn, 160–200° at 2° min^{-1}, 200–260° at 10° min^{-1}, 260° for 5 min. The MeOH phase was *N*-acetylated, then silylated and analysed by GC using the conditions stated above.

Lipid analysis of LPSs. Lipid analysis was also performed on the acid hydrolysis ppt. of LPSs. In this case, the ppt. obtained by centrifugation was esterified with 1M MeOH–HCl at 80° for 20 hr. After cooling, hexane was added to the reaction mixt. The hexane fr. was analysed by GC for lipid content.

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