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Characterization of lipids A of *Ralstonia solanacearum* lipopolysaccharides

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The analysis of fatty acid profiles of lipopolysacharides has shown that R. solanacearum strains tested may be divided into two groups. The first group is represented by R. solanacearum strains (5712, 7945, 7955 and 8110) the lipids A of which contained hydroxylated fatty acids with long chains: 3-hydroxytetradecanoic, 2-hydroxyhexadecanoic and 2-hydroxyoctadecanoic. The second group was represented by R. solanacearum strains the lipids A of which contained hydroxylated fatty acids with short chains: 3-hydroxydecanoic, 2-hydroxydodecanoic and 3-hydroxydodecanoic. 3-hydroxytetradecanoic acid was observed in a small amount. A comparative analysis of the fatty acid composition and biological activity gives a possibility to suppose that 3-hydroxytetradecanoic, 2-hydroxyhexadecanoic and 2-hydroxyoctadecanoic acids may be responsible for the toxicity and pyrogenicity of the lipopolysaccharides tested.

Introduction. There is a number of problems in classification of *Ralstonia solanacearum* which is a heterogeneous species. Up to 1992 the representatives of *R. solanacearum* were attributed to genus *Pseudomonas*. But during next 4 years Yabuuchi and coworkers twice reclassified species *solanacearum*. At first they transferred it to new genus *Burkholderia* [1] and then, in 1995 — to genus *Ralstonia* [2]. Whether this classification is the last, it is unknown. *R. solanacearum* is a very complex species. The heterogeneity of its strains remained to be solved taxonomically. The generally accepted criteria useful in taxonomy of gram-negative bacteria are the composition and structure of lipopolysaccharides (LPS), the components of outer membrane. Each of LPS components: O-specific polysaccharides (O-PS), oligosaccharide core and lipid A, is characterised by specific composition, displays different biological activity and has a various taxonomy significance. While fine O-PS structures are used as a basis for intraspecies classification schemes of gram-negative bacteria, lipid A is an endotoxic centre which is responsible for a majority of biological effects of LPS. So far as lipid A is the most conservative part of LPS molecule, its composition of fatty acids, in particular, hydroxylated ones, may be used as one of the additional taxonomic criteria. The purpose of present research was to investigate the fatty acid composition of lipids A of *R. solanacearum* strains and to study biological activity (toxicity and pyrogenicity) of the native and deacylated LPS.

Materials and Methods. *R. solanacearum* strains were kindly given by Dr. J. Young, curator of ICMP (New Zealand) (Table 1).

The bacterial cultures were grown on a synthetic medium [3] at 28 °C with shaking for 48 h.

The LPS were extracted from acetone- and ether-dried cells with 45 % aqueous phenol at 65—68 °C [4]. The aqueous layers were dialyzed against distilled water, nucleic acids were removed by precipitation with trichloroacetic acid and the solution was liophylized. For isolation of lipid A, the LPS were treated with 1 % acetic acid (100 °C, 2.0 h) and lipids A were obtained by ultracentrifugation (25000g, 40 min). The LPS were hydrolyzed in 1.5 % acetyl chloride in methanol (100 °C, 4 h) and methyl esters of fatty acids were analyzed by gas-liquid chromatography/mass spectrometry (Hewlett Packard, USA), equipped with computer assistance.

The O-deacylated LPS were obtained by alkaline hydrolysis (0.2 M NaOH in 99.0 % ethanol, 50 °C,

Table 1
Charactarization of R. solanacearum strains

ICMP strains	Geographic region	Biovar	Host-plant
5712	USA	I	Tomato
767	Trinidad	I	Banana
4157	New Zealand	—	Potato
7944	Peru	I	Plantain
7945	Peru	IV	Potato
7955	Kenya	III	Eggplants
8089	Philippines	II	Sweet pepper
8110	Sri-Lanka	IV	Potato

18 h). The hydrolyzate was neutralized, dialysed against water to remove the salt, and liophylized. The N-, O-deacylated LPS were obtained by treating with anhydrous hydrazine (1 ml) in a sealed tube for 40 h at 100 °C. After the reaction, excess hydrazine was diluted with water. The mixture was neutralized with HCl and the fatty acid hydrazides liberated were removed by extraction with chloroform. The deacylated LPS were obtained after eliminating the salt by gel-filtration [5].

The toxicity was studied by estimation of lethal toxicity (LD_{50}), using the galactosamine-sensitized mice, by injection of different concentrations (from 50 to 300 μg/ml) of native and modified LPS. The pyrogenicity was estimated by taking rabbits temperature after injection of different concentrations (from $0.5 \cdot 10^{-2}$ to $1.0 \cdot 10^{-2}$ mg/ml) of the native and modified LPS [6].

Results and Discussion. Lipid A is the most conservative part of the LPS molecule. It consists of 1,4'-biphosphorylated β-1,6-interlinked glucosamine-disaccharide with 4 residues of amide and esterlinked (R)-3-hydroxylated fatty acids which carry 2 or 4 nonhydroxylated acyl groups. Lipids A of different bacterial species vary from each other in the composition of fatty acids, in particular, hydroxylated ones, which is a stable index and therefore may be used as one of the chemotaxonomic criteria.

An analysis of fatty acid profiles of LPS showed that *R. solanacearum* strains tested may be divided into two groups (Table 2). The first group was represented by *R. solanacearum* strains (5712, 7945, 7955 and 8110) lipids A of which contained the hydroxylated fatty acids with long chains: 3-hydroxytetradecanoic, 2-hydroxyhexadecanoic and 2-hydroxyoctadecanoic. The second group was represented by *R. solanacearum* strains lipids A of which contained the hydroxylated fatty acids with short chains: 3-hydroxydecanoic, 2-hydroxydodecanoic and 3-hydroxydodecanoic. 3-hydroxytetradecanoic acid was observed in a small amount.

Wilkinson and coworkers [7] studied a number of strains of genus *Burkholderia cepacia*, to which *R. solanacearum* was applied up to 1995, and two *Ralstonia* species: *eurotropha* and *pickettii*. The comparative analysis of their fatty acids profiles indicates that *R. solanacearum* is closely relates to *B. cepacia* and contains 3-hydroxytetradecanoic and 3-hydroxyhexadecanoic acids in their lipids A. The investigated LPS from either *R. pickettii* or *R. eurotropha* strains, don't contain 3-hydroxydecanoic acid and 2-hydroxyoctadecenic acid (observed by some authors in *R. solanacearum* [8]). The presence of 3-hydroxydodecanoic acids is characteristic of lipids A of such phytopathogenic species as *Pseudomonas syringae* and *P. fluorescens*. The results obtained on heterogeneity of lipids A of *R. solanacearum* strains coincides with the data on heterogeneity of O-specific polysaccharide structures, on the basis of which the *R. solanacearum* strains tested were disributed into 5 serogroups (Table 3).

R. solanacearum is one of the most destructive bacterial pathogens, damaging a wide range of economically important plants such as potato, tomato, eggplants, banana, sweet pepper etc. Via the agricultural products *R. solanacearum* get into the warm-blooded organisms and may display toxicity. Therefore we studied the toxicity and pyrogenicity of the LPS of *R. solanacearum* strains investigated. According to the results obtained, the LPS may be divided into two groups one of which includes nontoxic and nonpyrogenic LPS (ICMP 767, 7944, 8089 and 4157), and the other one contains toxic and pyrogenic LPS (ICMP 5712, 8110, 7945 and 7955). To establish the chemical groups responsible for toxicity and pyrogenicity, the modified LPS, dephosphorylated and deacylated, were obtained from *R. solanacearum* 5712. It was shown that the LPS tested have lost the toxicity and pyrogenicity. These data indicate the lipid A acyl and phosphate groups are responsible for *R. solanacearum* LPS toxicity and pyrogenicity. The comparative analysis of fatty acid composition and biological activity gives a possibility to suppose that 3-hydroxytetradecanoic, 2-hydroxyhexadecanoic and 2-hydroxyoctadecanoic acids may be responsible for the toxicity and pyrogenicity of the LPS tested. The literature data on the correlation between the LPS biological activity and length of lipid A fatty acids are in favour of such suggestion. In particular, the LPS containing short chain fatty acids are less pyrogenic and toxic in comparison to long chain ones [9].

Table 2
Fatty acid composition of lipid A of R. solanacearum (% of the total peak area)

Fatty acid	ICMP strains						
	5712	7945	7955	8110	767	7944	8089
3-OH-$C_{10:0}$	—	—	—	—	—	—	18.23
$C_{12:0}$	0.81	0.07	0.53	0.93	1.07	19.76	16.21
2-OH-$C_{11:0}$	—	—	—	—	—	—	—
3-OH-$C_{11:0}$	—	—	—	—	—	3.0	1.14
2-OH-$C_{12:0}$	—	—	—	—	—	—	23.6
3-OH-$C_{12:0}$	0.74	0.44	0.28	0.36	0.85	25.64	18/12
$C_{14:0}$	19.22	16.63	20.64	16.21	4.19	3.17	1.85
2-OH-$C_{13:0}$	—	—	—	—	—	—	—
3-OH-$C_{13:0}$	0.16	—	1.71	0.15	—	—	—
i-$C_{15:0}$	0.11	—	—	—	18.1	1.47	0.62
ai-$C_{15:0}$	—	—	—	—	27.04	—	1.22
2-OH-$C_{14:0}$	—	—	2.17	1.7	—	—	—
3-OH-$C_{14:0}$	33.53	24.41	27.61	23.68	1.04	16.9	0.39
$C_{16:1}$	—	—	1.16	2.71	—	—	—
$C_{16:0}$	8.72	12.56	6.09	7.29	18.0	22.74	13.71
3-OH-$C_{15:0}$	—	1.38	1.35	2.66	—	—	—
ai-$C_{17:0}$	—	—	—	—	7.92	1.45	0.48
Isomeric-2-OH-$C_{16:0}$	—	2.67	—	—	—	—	—
2-OH-$C_{16:0}$	21.55	10.43	5.27	10.51	—	—	—
3-OH-$C_{16:0}$	1.09	0.82	0.63	1.43	3.44	1.63	—
cis-9-$C_{18:0}$	—	10.65	—		—	—	—
trans-9-$C_{18:1}$	—	—	5.07		—	—	—
$C_{18:1}$	2.66	2.37	8.7		17.45	4.24	4.43
2-OH-$C_{18:1}$	8.3	—	—		—	—	—
2-OH-$C_{18:0}$	3.12	17.56	17.32		—	—	—
3-OH-$C_{18:0}$	—	—	1.48		—	—	—

Table 3
Biological activity of R. solanacearum LPS

LPS, strain ICMP	Average values of temperature changes (°C) after LPS injection during			LD50	
	1 h	2 h	3 h	μg/mouse	g/kg
5712	+0.79	+0.80	+0.20	12	0.6
7945	+0.95	+0.5	−0.07	10	0.5
7955	+1.27	+1.21	+0.81	8	0.4
8110	+0.96	+0.55	+0.26	10	0.5
767	+0.15	+0.11	+0.01	35	1.75
7944	+0.30	0	−0.31	30	1.5
8089	+0.05	+0.26	−0.08	35	1.75
4157	+0.20	+0.20	0	40	2

N o t e. Minimal pyrogenic doze (rabbits) μg/ml — $7.5 \cdot 10^{-3}$.

Conclusions. Thus it has been shown the heterogeneity of *R. solanacearum* strains tested in the composition of their LPS fatty acids. The strains differ by the presence of hydroxylated fatty acids with short or long of chain.

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Характеристика ліпідів А ліпополісахаридів *Ralstonia solanacearum*

Резюме

Аналіз жирнокислотних профілів ліпополісахаридів свідчить про те, що досліджені штами R. solanacearum можна поділити на дві групи. Перша група представлена штамами R. solanacearum (5712, 7945, 7955 і 8110), ліпіди А яких містять оксикислоти з довгими вуглецевими ланцюгами: 3-окситетрадеканову, 2-оксигексадеканову та 2-оксиоктадеканову. В другу групу входять штами R. solanacearum, у ліпідах А яких присутні оксикислоти з короткими ланцюгами: 3-оксидеканова, 2-оксидодеканова та 3-оксидодеканова. 3-окситетрадеканову кислоту знайдено в незначній кількості. Порівняльний аналіз жирнокислотного складу та біологічної активності дає підставу припустити, що 3-окситетрадеканова, 2-оксигексадеканова та 2-оксиоктадеканова кислоти можуть відповідати за токсичність та пірогенність досліджуваних ліпополісахаридів.

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Характеристика липидов А липополисахаридов *Ralstonia solanacearum*

Резюме

Анализ жирнокислотных профилей липополисахаридов выявил, что исследованные штаммы R. solanacearum могут быть разделены на две группы. Первая группа представлена штаммами R. solanacearum (5712, 7945, 7955 и 8110), липиды А которых содержат оксикислоты с длинными цепями: 3-окситетрадекановую, 2-оксигексадекановую и 2-оксиоктадекановую. Во вторую группу входят штаммы R. solanacearum, в липидах А которых присутствуют оксикислоты с короткими цепями: 3-оксидекановая, 2-оксидодекановая и 3-оксидодекановая. 3-окситетрадекановая кислота обнаружена в незначительном количестве. Сравнительный анализ жирнокислотного состава и биологической активности дает основание предположить, что 3-окситетрадекановая, 2-оксигексадекановая и 2-оксиоктадекановая кислоты могут отвечать за токсичность и пирогенность исследованных липополисахаридов.

REFERENCES

1. *Yabuuchi E., Kosako Y., Oyaizu H., Yano I., Hotta H., Hashimoto Y., Ezaki T., Arakawa.* Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes, 1981) comb. nov // Microbiol. Immunol.—1992.—**136**.—P. 1251—1275.
2. *Yabuuchi E., Kosako Y., Yano I., Hotta H., Nichiuchi Y.* Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: Proposal of *Ralstonia pickettii* (Ralston, Palleroni, Doudoroff, 1973) comb. nov. and *Ralstonia eurotropha* (Davis, 1969) comb. nov // Microbiol. Immunol.—1995.—**39**.—P. 897—904.
3. *Vidaver A.* Synthetic and complex media for the rapid detection of fluorescence of phytopathogenic *pseudomonas*: effect of the carbon source // Appl. Microbiol.—1967.—**15**.—P. 1523—1524.
4. *Westphal O., Jann K.* Bacterial lipopolysaccharide exctraction with phenol-water and further application of the procedure // Meth. in Carbohydrate Chem. / Eds R. Whistler, M. Wolfrom.—New York: Acad. press, 1965.—Vol. 5.—P. 83—91.
5. *Tanamoto K., Ichibashi N.* Succinilated lipid A as a potent specific inhibitor of endotoxin mitogenicity // J. Gen. Microbiol.—1992.—**138**.—P. 2503—2508.
6. *Takahashi K., Morikawa A., Kato Y.* Flavonoids protect mice from two types of lethal shock induced by endotoxin // FEMS Immunol. Microbiol.—2001.—**31**.—P. 29—33.
7. *Galbraith L., Jonsson M., Rudhe L., Wilkinson S.* Lipids and fatty acids of *Burkholderia* and *Ralstonia* species // FEMS Microbiol. Lett.—1999.—**173**.—P. 359—364.
8. *Akiyama Y., Nishikawaji S., Eda S., Tanaka H., Ohnishi A., Kato K.* Lipopolysaccharide of *Pseudomonas solanacearum* // Agr. Biol. Chem.—1985.—**49**.—P. 1193—1194.
9. *Branderburg K., Mayer H., Koch M., Weckesser J., Rietschel E., Seydel U.* Influence of supramolecular structure of free lipid A on its biological activity // Eur. J. Biochem.—1993.—**218**.—P. 555—563.

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