Comparative Studies on Fatty Acid Composition of the Whole-Cell and Outer Membrane in Brazilian Strains of Ralstonia solanacearum

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Comparative Studies on Fatty Acid Composition of the Whole-Cell and Outer Membrane in Brazilian Strains of *Ralstonia solanacearum*

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The fatty acid analysis of Brazilian strains of *Ralstonia solanacearum* was conducted by gas–liquid chromatography (GLC). Using 29 strains from Brazil, profiles of the whole-cell fatty acid methyl esters (FAME) were compared. Qualitative and quantitative differences in the profiles of FAMEs were not observed among the strains.

When the fatty acid analysis of the bacterial cellular membrane was conducted, striking outcome was obtained. In particular, the ratios of the amount of 2–hydroxypalmitic acid(16:1 2–OH) and an unidentifited fatty acid (Rt 16.4) varied greatly depending on original host plants and biovars. The fatty acid profiles of the strains from pepper (biovars 2 and 3), banana (biovar 1) and eucalyptus (biovar 1) were clearly different from those of the strains isolated from other plants. These results indicate that fatty acid profiles of the outer–membrane might reflect the differences of the host plants from which the isolates were obtained. Bacterial outer–membrane fatty acid profiles will be useful as a benchmark for the classification and identification of *R. solanacearum* at subspecies level.

INTRODUCTION

Intraspecific grouping of *Ralstonia solanacearum* (Yabuuchi *et al.*, 1995) (syn. *Pseudomonas solanacearum*) remains a complex subject (Okabe and Goto, 1961). Arrangements based on host specificity, which resulted in five races (Buddenhagen and Kelman, 1964, Buddenhagen *et al.*, 1962, He *et al.*, 1983), did not coincide with grouping based on physiological criteria (Hayward, 1964, He *et al.*, 1983), which lead to the recognition of five biovars. Further detailed phenotypic, chemotaxonomic and genetic studies are required to clarify the taxonomic structure of *R. solanacearum*.

To supply additional data which may help to understand the complex species of *R. solanacearum*, we analyzed the whole–cell and the outer–membrane fatty acid methyl esters (FAMEs) of Brazilian strains of this bacterium by gas–liquid chromatography.

MATERIALS AND METHODS

Bacterial isolates and culture

Twenty–nine Brazilian strains of *Ralstonia solanacearum* and type strain ATCC11696 and type strains of *R. pickettii* ATCC27511, *Erwinia carotovora* subsp.

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carotovora ATCC33260, *E. c.* subsp. *atroseptica* ATCC43762, *Xanthomonas campestris* pv. *campestris* ATCC33913, *Burkholderia* (Yabuuchi *et al.*, 1992) (syn: *Pseudomonas*) *caryophylli* ATCC25418, *B. gladioli* pv. *gladioli* ATCC10248 and *Agrobacterium tumefaciens* ATCC23308 maintained in author's laboratory were used in this study. Each bacterial strain of *R. solanacearum* was pre-cultured on the plate of TTC medium (peptone 10 g, casein hydrolysate (Difco) 1 g, glucose 5 g, agar 17 g, triphenyl tetrazolium chloride (1% solution) 5 ml, distilled water 1 liter) at 30 °C for 48 hr for selecting virulent colonies. Typical virulent colonies were isolated and cultured in 200 ml of 523 medium (Kado and Heskett, 1970) in Sakaguchi flask by shaking at 30 °C for 24 hr. Type strains other than *R. solanacearum* were pre-cultured on the plates of potato semi-synthetic agar (PSA) medium (Wakimoto, 1955) at 30 °C for 24 hr and then cultured in 523 broth at 30 °C for 24 hr.

Preparation of the samples

The bacterial cells were harvested by centrifugation (3,500×*g*, 30 min), resuspended in 0.85% NaCl solution and centrifuged. A part of the pellet was lyophilized and stored as the whole-cell sample. Five grams (f.wt) of the precipitated cells was resuspended in 100 ml of 0.2 M LiCl solution and shaken (156 strokes/min) at 45 °C for 2.5 hr with glass beads (1 mm in diameter). The supernatant obtained by centrifugation (5,000×*g*, 20 min) was recentrifuged at 30,000×*g* for 40 min to remove large membranous materials. The resulting supernatant was then centrifuged at 100,000×*g* for 2 hr and the pellet was washed once with distilled water under the same centrifugal conditions. All of the centrifugation was conducted at 4 °C. The bacterial outer membrane obtained was lyophilized and stored in a desiccator.

Preparation of fatty acids

Five milligrams of the lyophilized whole-cell or outer membrane was methylated with 2 ml of 5% HCl-methanol at 100 °C for 3 hr in a sealed glass tube to obtain fatty acid methyl ester (FAME) derivatives. After methanolysis, one ml of water was added and the FAMEs were extracted with petroleum ether by shaking. The solvent phase was washed with equal volume of distilled water to remove HCl and dehydrated by mixing with 0.5 mg of anhydrous sodium sulfate. The organic phase was concentrated by nitrogen gas blowing. Samples were stored at -20 °C.

Preparation of fatty acids

FAMEs were analyzed by a gas-liquid chromatograph (Shimazu GC 17A) equipped with a FID detector and 0.25 mm×50 m HR-SS-10 capillary column. The column and injection-port temperatures were maintained at 180 °C and 250 °C, respectively. The pressure of nitrogen gas was 95 Kpa. Each FAME was identified by comparing its retention time with known samples. Peak area was calculated automatically and expressed as percentage composition. The analysis was repeated three times for each strain. Average values of the composition of fatty acids were used to differentiate the strains of *R. solanacearum*.

RESULTS AND DISCUSSION

The results in Table 1 show that various species of phytopathogenic bacteria have unique FAMEs profiles. All of the strains contained myristic (14:0), palmitic (16:0) and palmitoleic acids (16:1 *cis* 9) and these acids were often major. Striking differences in the composition of fatty acids were observed among type strains of *Ralstonia solanacearum*, *Erwinia carotovora* subsp. *atroseptica*, *E. c.* subsp. *carotovora*, *Agrobacterium tumefaciens* and *Xanthomonas campestris* pv. *campestris*. Moreover, *R. solanacearum* was differentiated from *Burkholderia caryophylli* and *B. gladioli* pv. *gladioli*, which belong to ribosomal RNA group II by DNA–DNA hybridization studies by Palleroni *et al.* (1973), on the basis of fatty acid compositions of oleic (18:1) and two kinds of unidentified fatty acids (Rt 8.9 and Rt 10.9). Although *R. pickettii* is very closely related with *R. solanacearum* in bacterial properties, these two species were readily differentiated by the presence or absence of oleic (18:1), 2–hydroxypalmitic (16:1 2–OH) and three kinds of unidentified fatty acids (Rt 8.9, Rt 11.2 and Rt 16.4).

Eight kinds of fatty acid were identified and quantified in Brazilian strains of *R. solanacearum*. They were myristic (14:0), palmitic (16:0), palmitoleic (16:1 *cis* 9), vaccenic (18:1 *cis* 11), 2–hydroxypalmitic (16:0 2–OH) and three kinds of unidentified fatty acids (Rt 9.7, Rt 10.9 and Rt 16.4).

As can be seen in Table 2, all of Brazilian strains had very similar profiles of whole–cell FAMEs and no significant differences were observed. In the case of outer membrane FAMEs indicated in Table 3, the unidentified fatty acid (Rt 16.4) was not found excepting eucalyptus strains. Although striking differences were not detectable within Brazilian strains, small but distinct differences were observed among the strains from different hosts and biovars. 2–hydroxypalmitic (16:1 2–OH) acid could not be detected in pepper strains of biovars 2 and 3. On the other hand, banana strain had a higher percentage of this fatty acid. The unidentified fatty acid (Rt 9.7) was detected at a high concentration in eggplant strains of biovar 2, cucumber strains of biovar 1, and pepper strains of biovars 1 and 3. These results might indicate that the FAME profiles of outer membrane related partly with the host or biovar. Further studies with larger numbers of strains and analysis under different cultural conditions will be required to determine whether *R. solanacearum* can be differentiated at biovar or race level by their fatty acid profiles.

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Table 1. Percentage composition of total cellular fatty acids in various type strains of phytopathogenic bacteria.

Type strain	Percentage composition of fatty acid															
	10:0	12:0	14:0	16:0	16:1 *cis*9	12:0 3 OH	Rt[a] 8.9	Rt 9.7	18:1	Rt 10.9	Rt. 11.2	18:1 *cis*11	20:0	16:1 2–OH	Rt 16.4	Rt 20.4
E. c. subsp. *atroseptica*	0.0[b]	6.97	4.56	33.58	21.94	0.00	2.89	0.00	5.03	0.00	11.97	0.00	13.03	0.00	0.00	0.00
E. c. subsp. *carotovora*	0.00	19.17	2.63	19.05	20.41	0.00	0.59	0.00	4.93	0.00	2.45	0.00	30.73	0.00	0.00	0.00
X. c. pv. *campestris*	17.70	0.00	10.09	15.18	32.47	14.94	7.23	0.00	0.00	0.00	0.00	2.35	0.00	0.00	0.00	0.00
B. caryophylli	0.00	0.00	5.03	22.45	10.61	0.00	3.06	0.00	14.79	0.00	8.20	18.03	0.00	4.13	2.14	11.51
B. g. pv. *gladioli*	0.00	0.00	6.59	19.34	14.07	0.00	0.88	0.00	10.41	0.00	7.46	20.36	0.00	3.34	4.42	13.07
A. tumefaciens	0.00	4.84	1.59	9.99	10.23	0.00	0.00	0.00	57.44	0.00	0.00	8.84	0.00	0.00	0.00	7.04
R. pickettii	0.00	0.00	7.81	15.54	32.40	0.00	3.89	0.00	6.50	0.00	1.86	31.96	0.00	0.00	0.00	0.00
R. solanacearum	0.00	0.00	11.68	20.18	10.15	0.00	0.00	4.96	0.00	7.38	0.00	23.82	0.00	19.10	2.69	0.00

a) Rt means retention time and Rt 8.9, Rt 9.7, Rt 10.9, Rt 11.2, Rt 16.4 and Rt 20.4 are unidentified fatty acids.

b) Fatty acids in each strain are expressed as a percentage of total cellular fatty acid compositions. Fatty acids were identified by their retention times on the gas-liquid chromatogram.

Table 2. Percentage composition of fatty acids of whole-cell in Brazilian strains of *Ralstonia solanacearum*.

Strain	Host	Biovar	Percentage composition of fatty acid							
			14:0	16:0	16:1 *cis*9	Rt[a] 9.7	Rt 10.9	18:1 *cis*11	16:1 2-OH	Rt 16.4[a]
578	Potato	1	11.0[b]	24.65	20.53	10.45	6.46	14.60	2.84	9.41
1005	Potato	1	9.40	31.94	14.46	9.77	8.73	17.18	3.93	4.55
98	Potato	2	9.10	19.17	34.12	8.64	4.26	18.96	0.85	4.87
964	Potato	2	9.74	20.20	27.15	8.69	4.29	22.39	3.87	3.63
799	Potato	2	11.16	31.42	25.69	12.69	4.65	9.96	2.31	2.08
19	Tomato	1	17.09	24.86	22.83	7.98	6.93	14.28	3.84	2.15
76	Tomato	1	9.86	33.72	29.20	15.37	2.79	6.41	1.07	1.53
1033	Tomato	1	11.08	24.33	19.02	7.45	11.05	18.69	2.73	5.61
855	Tomato	2	12.73	25.13	27.79	14.75	6.69	8.42	2.71	1.74
49	Tomato	3	12.93	23.19	30.28	11.32	2.90	10.96	2.70	5.67
630	Tomato	3	7.17	30.44	28.37	17.88	3.52	8.22	1.68	2.68
628	Tomato	3	7.83	38.25	27.40	18.71	3.34	3.45	0.77	0.21
1104	Tomato	3	6.88	25.81	17.00	9.02	13.15	20.09	2.73	5.28
127	Pepper	1	11.67	37.65	24.12	15.96	3.33	4.66	1.30	1.27
162	Pepper	2	14.08	26.61	27.09	12.47	5.44	9.83	0.85	3.58
7	Pepper	2	9.71	23.29	23.71	14.35	2.42	10.40	11.98	4.10
20	Pepper	3	9.60	30.92	31.04	12.69	3.88	9.04	0.53	2.24
582	Pepper	3	9.70	23.71	32.32	14.34	3.54	12.46	1.50	2.39
73	Banana	1	7.08	29.72	28.73	12.14	3.56	15.25	2.06	1.40
656	Cucumber	1	10.80	26.65	27.65	7.62	4.59	6.18	14.87	1.61
129	Cucumber	1	9.29	24.31	29.42	9.31	4.12	19.20	3.00	1.30
574	Eucalyptus	1	12.20	25.86	31.10	8.88	5.67	10.53	4.36	1.36
579	Eucalyptus	1	12.96	22.66	28.18	7.87	5.72	16.91	2.08	3.58
87	Eggplant	2	11.58	22.68	23.19	8.17	6.19	19.27	5.48	3.42
79	Eggplant	2	10.85	20.84	26.52	8.15	5.45	21.26	3.22	3.68
71	Eggplant	2	18.12	25.69	28.62	10.34	5.99	8.17	1.22	1.82
56	Eggplant	3	14.32	26.53	30.73	10.31	5.22	9.83	0.61	2.41
51	Eggplant	3	7.57	24.61	34.60	14.67	3.51	11.03	0.86	3.12
47	*Solanum gilo*	3	16.36	33.75	11.85	12.59	3.90	11.16	6.20	4.15

a) Rt means retention time and Rt 9.7, Rt 10.9 and Rt 16.4 are unidentified fatty acids.

b) Fatty acids in each strain are expressed as a percentage of total outer membrane fatty acid compositions. Fatty acids were identified by their retention times on the gas-liquid chromatogram.

Table 3. Percentage composition of fatty acid of bacterial outer membrane in Brazilian strains of *Ralstonia solanacearum*.

Strain	Host	Biovar	Percentage composition of fatty acid							
			14:0	16:0	16:1 *cis*9	Rt[a] 9.7	Rt 10.9	18:1 *cis*11	16:1 2-OH	Rt 16.4
578	Potato	1	20.62[b]	24.08	11.87	7.63	3.21	30.19	2.37	0.00
1005	Potato	1	17.59	13.07	22.18	2.34	1.79	42.18	0.83	0.00
98	Potato	2	12.80	22.43	19.18	10.10	2.72	27.33	5.40	0.00
964	Potato	2	16.26	14.37	25.63	1.92	2.03	38.53	1.23	0.00
799	Potato	2	18.15	17.32	26.83	3.44	2.15	32.07	0.00	0.00
19	Tomato	1	19.59	11.92	20.96	2.03	1.74	42.05	1.68	0.00
76	Tomato	1	15.43	34.09	18.48	0.04	5.82	26.09	0.02	0.00
1033	Tomato	1	15.11	17.42	23.47	3.85	3.07	37.04	0.00	0.00
855	Tomato	2	15.83	14.49	13.33	5.55	2.68	44.79	3.30	0.00
49	Tomato	3	20.50	12.04	38.20	1.12	1.40	22.07	4.27	0.00
630	Tomato	3	14.44	9.76	26.43	5.01	1.11	42.29	0.93	0.00
628	Tomato	3	12.53	11.84	27.58	5.88	0.72	41.42	0.00	0.00
1104	Tomato	3	17.57	16.24	22.90	0.00	2.05	38.68	2.54	0.00
127	Pepper	1	14.32	23.33	16.13	12.37	2.71	30.32	0.79	0.00
162	Pepper	2	22.13	18.56	37.02	1.06	0.87	20.34	0.00	0.00
7	Pepper	2	22.60	11.53	4.35	1.67	15.20	44.62	0.00	0.00
20	Pepper	3	15.11	16.57	19.25	14.13	0.82	34.10	0.00	0.00
582	Pepper	3	12.99	18.65	17.98	15.95	2.68	31.72	0.00	0.00
73	Banana	1	22.23	16.67	23.89	4.01	1.98	18.81	12.39	0.00
656	Cucumber	1	9.19	27.77	27.76	13.07	1.86	17.97	2.35	0.00
129	Cucumber	1	14.18	24.70	19.56	16.59	3.79	21.14	0.01	0.00
574	Eucalyptus	1	18.42	11.67	31.89	0.18	0.60	34.62	1.28	1.29
579	Eucalyptus	1	11.35	13.21	19.66	7.37	1.35	46.11	0.80	0.12
87	Eggplant	2	11.98	28.83	16.14	16.72	4.20	21.40	0.69	0.00
79	Eggplant	2	15.15	20.06	31.38	9.45	1.95	21.98	0.00	0.00
71	Eggplant	2	10.94	18.75	19.17	12.87	3.32	33.91	1.01	0.00
56	Eggplant	3	20.90	16.09	32.95	1.49	1.19	27.35	0.00	0.00
51	Eggplant	3	15.35	14.83	23.95	4.80	1.29	37.45	2.30	0.00
47	*Solanum gilo*	3	16.33	14.31	26.52	4.47	1.76	35.97	0.60	0.00

a) Rt means retention time and Rt 9.7, Rt 10.9 and Rt 16.4 are unidentified fatty acids.

b) Fatty acids in each strain are expressed as a percentage of total outer membrane fatty acid compositions. Fatty acids were identified by their retention times on the gas–liquid chromatogram.

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