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Differentiation of *Erwinia chrysanthemi* and *E. carotovora* subsp. *carotovora* by the Cellular Fatty Acid Analysis

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For the accurate differentiation of erwinia, the fatty acid analysis of the bacterial cellular membrane of *Erwinia* species was conducted by gas-liquid chromatography. Striking outcome was obtained in the case of comparison between *E. chrysanthemi* isolates and *E. carotovora* subsp. carotovora. The chromatograms of two *Erwinia* species were distinctly different. The ratios of the amount of lauric acid (12:0) and myristic acid (14:0) in these two species were reverse each other. In *E. chrysanthemi* pv. *zeae*, the fatty acid profile of isolate R7 and R8 from rice was clearly different from that of corn isolate. This result indicates that the fatty acid profile might reflect the differences of the host from which the isolate was obtained. Fatty acids profile will be useful as a benchmark for the classification and identification of phytopathogenic bacteria.

INTRODUCTION

Differentiation of plant pathogenic bacteria has mainly been conducted on the basis of the morphological, physiological and pathological characteristics. In addition to these criteria, the data from serological and chemical analysis of the bacterial cells have been involved, recently (Yaurks and Shaad, 1979). Especially, the analysis of cellular fatty acid composition has been stressed in bacteria such as clavibacter, pseudomonads and some of erwinias (Ikemoto et al., 1978, Suzuki and Komagata, 1983, De Boer and Sasser, 1986).

De Boer and Sasser (1986) analyzed the cellular fatty acids of ***Emvinia carotovora,*** the enteric bacterial species, which causes soft rot in many different plants by gas–liquid chromatography. They reported that two subspecies, spp. *carotovora* and *atroseptica*, could be differentiated on the basis of three different fatty acid ratios. In previous report, the authors reported that electrophoretic profiles of the outer membranous proteins of ***Emvinia chrysanthemi*** isolates were different at pathovar level and sometimes related with hosts from which each isolate was obtained (Uesugi et al., 1990). To examine if the differentiation of pathovars of ***Emvinia chrysanthemi*** would also be available by the fatty acid analysis, the following experiments were conducted.

MATERIALS AND METHODS

Bacterial isolates and culture

Twenty isolates of ***E. chrysanthemi*** and 4 isolates of ***E.*** *carotovora* subsp. *carotovora* maintained in author's laboratory (Table 1) were used in this study. Bacterial isolates were precultured on the plates of YPA medium (5g yeast extract, 10g peptone, 5g

NaCl, 15g agar, dist. water 1 1, pH 6.8) at 30°C for 24hr and then cultured in 200ml of 523 medium (Kado and Heskett, 1970) in Sakaguchi flask by shaking at 30°C for 24 hr.

Preparation of the samples

The bacterial cells were harvested by centrifugation (3,500 Xg, 30min), resuspended in 0.85% NaCl solution and centrifuged. Five grams (f.wt.) of the precipitated cells were resuspended in 100ml of 0.2M LiCl solution and shaked (156 strokes/min) at 45" C for 2.5 hr with 7.5g glass beads (lmm in diameter). The supernatant obtained by centrifugation (5,000 *x g,* 20min) was recentrifuged at 30,000 Xg for 40min to remove large membranous materials. The resulting supernatant was then centrifuged at 100,000 Xg for 2hr and the pellet was washed once with distilled water under the same centrifugal condition. All of the centrifugation were conducted at 4°C. The bacterial outer membrane obtained was lyophilized and stored in a desiccator.

Analysis of fatty acids by GLC

Five milligrams of the lyophilized outer membrane was methylated with 2ml of 5% HCl-methanol at 100°C for 3 hr. After methanolysis, lml of water was added and

Table 1. Bacterial strains used in this study.

Bacterium	Strain	Isolated from
Erwinia chrysanthemi pv. *chrysanthemi*	Ku 8601"	Pear
〃 〃 〃	Ichihara 1-1[2]	Pear
〃 〃 〃	E 8301[3]	Chrysanthemum
〃 〃 pv. *zeae*	R7[3]	Rice
〃 〃 〃	R8[3]	Rice
〃 〃 〃	511-3"	Corn
〃 〃 〃	Corn 801[3]	Corn
〃 〃 〃	Corn 802[3]	Corn
〃 〃 〃	Corn 803[3]	Corn
〃 〃 〃	ALE 8292p[3]	Welsh onion
〃 〃 〃	NCPPB 377[5]	Corn
〃 〃 pv. *dian thicola*	Dianth 2n[3]	Carnation
〃 〃 〃	Dianth 1e[3]	Carnation
Erwinia chrysanthemi pv. unidentified	Ech 33"	Taro
〃 〃 〃	Ech 44[1]	Taro
〃 〃 〃	342-15[1]	Onion
〃 〃 〃	342-16S[1]	Onion
〃 〃 〃	329-21[1]	Mung bean
〃 〃 〃	329-23[1]	Mung bean
〃 〃 〃	E 7188[4]	Potato
Erwinia carotovora subsp. *carotovora*	57	Chinese cabbage
〃 〃 〃	B-1	Brocoli
〃 〃 〃	T-1	Chinese cabbage
〃 〃 〃	N7101	Sweet pepper

1) Plant Pathology Laboratory, Kyushu University.
2) From Dr. K. Suyama, Tokyo Agricultural University.
3) From Dr. M. Goto, Shizuoka University.
4) From Dr. K. Tsuchiya, National Institute of Agro-Environmental Sciences, Tsukuba, Ibaraki.
5) Purchased from NCPPB.

fatty acids were extracted with petroleum ether for 3 times. The solvent fraction was washed with the same volume of distilled water to remove HCl, dehydrated with anhydrous Na_2SO_4, concentrated by N_2 gas blowing and subjected to GLC (Shimazu GC-7AG,FID, column:10% DEGS 0.45cm x 3m, column temp.:180°C,N_2 flow rate:50ml/min). Fatty acids were identified by the comparison of retention time(Rt) with the standard. The percentage of each fatty acid was automatically calculated by the integrator (Shimazu C-R1A).

RESULTS AND DISCUSSION

Ten kinds of fatty acids were detected in the bacterial outer membrane of *E. chrysanthemi* and *E. carotovora* subsp. *carotovora*. Lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1), oleic acid (18:1) and arachidic acid (20:0) were detected in all isolates. Striking difference in the composition of fatty acids was observed between *E. carotovora* subsp. *carotovora* strains and ***E. chrysanthemi*** strains. In the case of ***E. carotovora*** subsp. ***carotovora,*** higher amount of lauric acid and lower amount of myristic acid were characteristically detected and this trend was vice versa in the case of ***E. chrysanthemi*** isolates (Fig. 1, Table 2). These results showed that ***E. chrysanthemi*** and *E. carotovora* will be differentiated clearly by the fatty acid profile. Especially the ratio of lauric acid and myristic acid will be useful markers. Although such striking difference was not detectable among pathovars of *E. chrysanthemi,* small but distinct differences were observed between pv. ***dianthicola*** and other pathovars. In the case of two isolates of ***E. chrysanthemi*** pv. *dianthicola,* higher palmitoleic acid content and no detection of two kinds of unidentified fatty acids (peaks 5,8 in Fig. 1) were characteristically observed.

In pv. *zeae,* the isolates R7 and R8 from rice were obviously different from the corn isolates. The amounts of unidentified fatty acid (peak 5), arachidic acid and palmitic acid of isolates R7 and R8 were specifically different from those of other isolates. This result might indicate that the fatty acid profile could partly relate with the host from which the isolate was obtained.

Although the similar electrophoretic profiles of cellular membranous protein were obtained among the isolated of pv. ***zeae*** (Uesugi ***et al.*** 1990), distinct difference was observed in the fatty acid composition.

Among the isolates of unidentified pathovar in ***E. chrysanthemi,*** the fatty acid profile of isolates 342-15 and 342-16S from onion were similar to the isolate ALE 8298p from Welsh onion, which belongs to pv. ***zeae.*** The resemblance was also observed between the isolate E 7188 from potato and two isolates of pv. ***dianthicola.*** However, it is uncertain if the isolates 342-15, 342-16S would be pv. *zeae* and E 7188 would be pv. ***dianthicola,*** respectively.

Although the existence of hydroxy fatty acid (3-OH, 14:0) in ***E. carotovora*** pv. ***carotovora*** has been reported (De Boer and Sasser, 1986), it was not detected in our experiment. Revision by the different extraction procedures and detection with capillary column at GLC will be required. Some peaks of the unidentified fatty acids such as peak 5,8 and 9 seemed to be the keys of the differentiation. The identification of these substances by the gas chromatography-mass spectrometry (GC-MS) will be requested.

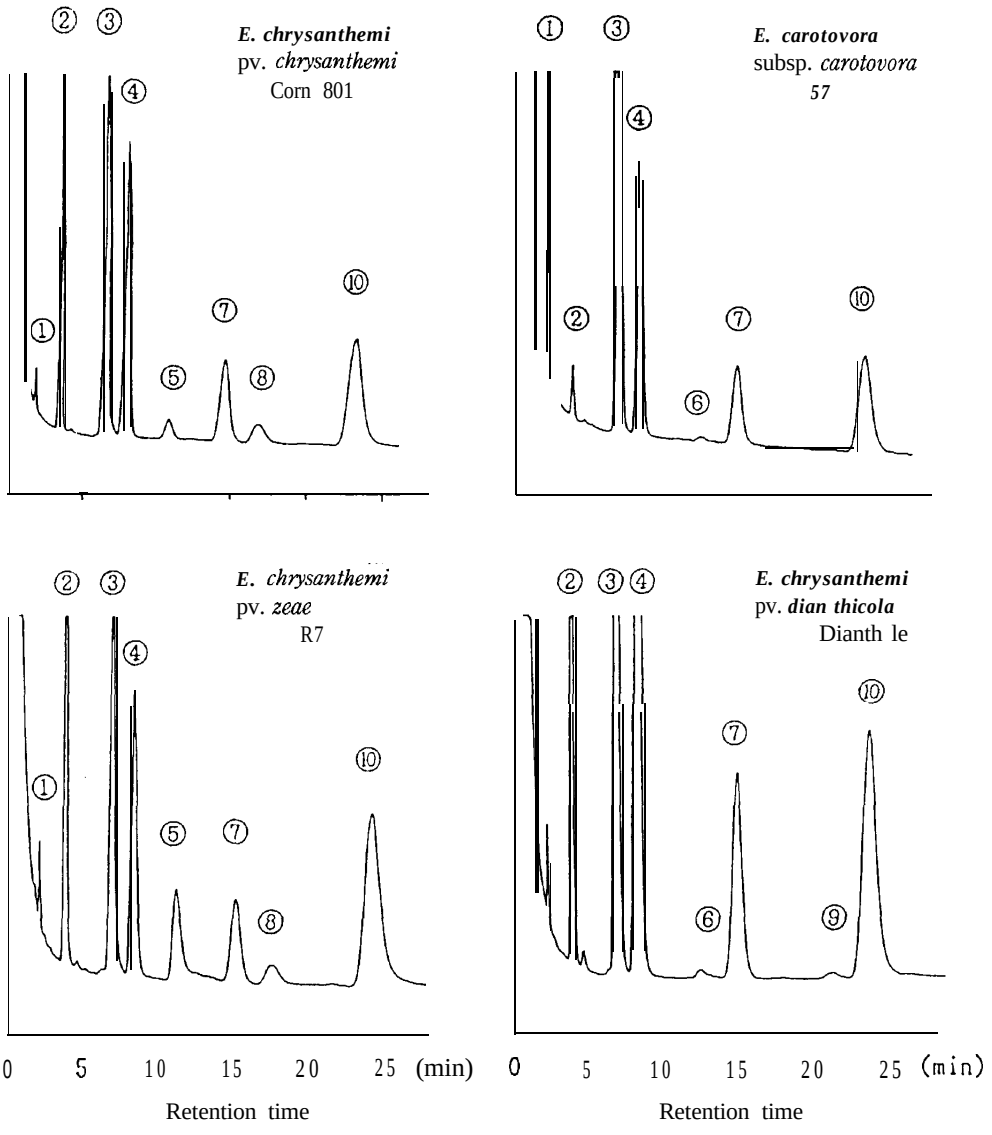

Fig. 1. Gas-liquid chromatograms of fatty acid of bacterial outer membrane in *Erwinia chrysanthemi* and *E. carotovora* subsp. *carotovora*. Numbered peaks represent the fatty acids as follows:
① Lauric acid (12:0),② Myristic acid (14:0),③ Palmitic acid (16:0),④ Palmitoleic acid (16:1),⑥ Stearic acid (18:0),⑦ Oleic acid (18:1),⑩ Arachidic acid (20:0),⑤,⑧,⑨ Unknown.

Table 2. Fatty acid of bacterial outer membrane in *Erwinia* species.

Strain	Fatty acid[1] and Amount									
	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩
E. chrysanthemi pv. ***chrysanthemi***										
Ku ***8601***	0.9[2]	17.2	29.3	15.8	2.3	ND	12.0	2.0	ND	20.5
Ichihara l-l	1.4	18.2	23.0	20.1	0.9	ND	12.6	1.1	ND	22.6
E 8301	0.8	14.3	25.9	29.6	0.4	ND	10.8	ND	ND	18.2
E. chrysanthemi pv. zeae										
R7	1.2	17.4	19.4	15.6	8.8	ND	7.8	2.4	ND	27.2
R8	1.3	18.3	20.1	14.8	6.6	ND	8.5	3.0	ND	27.2
511-3	1.3	15.1	28.8	16.4	3.0	0.6	7.8	2.3	ND	25.1
Corn 801	0.9	14.2	25.6	22.3	2.1	ND	10.6	3.5	ND	20.7
Corn 802	0.9	16.5	24.8	22.1	1.5	ND	12.4	1.5	ND	20.4
Corn 803	0.9	16.6	28.3	19.0	2.2	ND	9.8	2.4	ND	20.8
ALE 8292p	0.9	16.1	30.8	18.6	1.6	ND	13.5	1.4	ND	17.1
NCPPB 377	1.8	21.0	24.4	9.5	5.4	ND	9.2	4.4	ND	24.3
E. chrysanthemi pv. ***diathicola***										
Dianth 2n	0.6	13.6	28.1	31.7	ND	ND	7.3	ND	ND	18.8
Dianth le	0.9	12.4	23.8	31.7	ND	0.4	10.9	ND	0.4	19.5
E. chrysanthemi pv. unidentified										
Ech 33	1.1	17.6	23.9	20.0	1.0	0.4	12.8	1.5	ND	21.9
Ech 44	0.8	17.2	27.7	20.4	0.9	0.6	13.6	0.9	ND	18.0
342-15	0.9	16.6	30.9	19.0	2.6	0.5	13.0	1.2	ND	15.3
342-16-S	0.9	14.2	25.8	25.5	1.0	0.3	16.3	0.5	ND	16.3
329-21	2.0	20.8	19.1	18.7	0.9	ND	7.5	1.1	ND	30.0
329-23	1.6	20.5	23.0	19.1	1.3	ND	8.4	1.3	ND	24.9
E 7188	1.0	13.0	25.3	30.7	0.5	0.7	12.7	0.4	ND	16.1
E. carotovora subsp. ***carotovora***										
57	15.0	1.9	33.4	20.4	ND	0.6	10.5	ND	ND	18.8
B-l	14.7	3.0	31.2	18.8	ND	ND	10.3	ND	ND	22.0
T-l	15.2	1.5	29.0	27.2	ND	ND	9.0	ND	ND	18.0
N 7101	14.4	1.9	37.0	19.0	ND	ND	11.0	ND	ND	16.7

1) ①=Lauric acid, ②=Myristic acid, ③=Palmitic acid, ④=Palmitoleic acid
⑥ = Stearic acid, ⑦ = Oleic acid, ⑩ = Arachidic acid, ⑤,⑧,⑨= unknown
2) Percentage of fatty acid in each strains.
ND: not detected.

Although further work with more isolates would be needed, the results obtained in this experiment will suggest the usefulness of the analysis of fatty acids of bacterial cellular membrane for the identification and classification of bacteria.

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