Detection of Antigen Determinants and Peptide Mapping of the Membrane Proteins of *Erwinia chrysanthemi* Pathovars

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Abstract

Erwinia chrysanthemi strains isolated from various hosts were serologically grouped with three antisera prepared against *E. chrysanthemi* strains Ku8601 isolated from pear, R8 from rice and 511-3 from corn. The strains isolated from carnation, pear, potato, onion, taro and welsh onion reacted with anti-Ku8601-serum, which were classified as serogroup I. Rice and corn strains respectively reacted with anti-R8 and anti-511-3 sera, showing high specificity which were classified as serogroup II and serogroup III. Membrane proteins of the bacterial strains belonging to each serogroup were separated with SDS-PAGE and immunoblotted on nitrocellulose sheet to determine antigenic proteins. The strains of serogroup I commonly possessed antigenic polypeptides of 38.5 and 5.9 kd that were considered to be serogroup-specific antigens. While the strains of serogroup II possessed antigenic polypeptides of 41.7 and 11.2 kd and those of serogroup III possessed 41.7, 40.3, 21.4 and 10.2 kd, respectively. Close relationship was observed between serological property and host of origin only in rice and corn strains.

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Key words: *Erwinia chrysanthemi*, membrane protein, antigen determinant, serology, peptide mapping, host of origin.

INTRODUCTION

On the basis of pathogenicity, *Erwinia chrysanthemi* strains were divided into six pathovars⁵⁾ such as pv. *chrysanthemi*, pv. *zeae*, pv. *dieffenbachiae*, pv. *partheni*, pv. *paradisiaca* and pv. *dianthicola*. In the previous paper we reported that pattern of membrane proteins of *E. chrysanthemi* revealed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were associated with host of origin²⁴⁾. Dickey and his co-workers^{3,4)} carried out serological studies with whole cells of *E. chrysanthemi* strains and indicated no specific relationship between serological properties and host of origin. Agglutination tests with membrane protein complex were reported to be useful only for differentiating *E. chrysanthemi* from other *Erwinia* spp.²⁵⁾ Membrane proteins extracted by LiCl solution were anteriorly used to identify serotype-specific antigen determinants of *Neisseria meningitidis*⁶⁾ and to characterize the subspecies-specific antigen determinants of *Xanthomonas campestris* pv. *campestris*²²⁾.

The purposes of this study were to find serogroup-specific antigen determinants in SDS-PAGE-separated membrane proteins or polypeptides of *E. chrysanthemi* strains isolated from various host plants and to clarify the relationship between antigen determinants and host of origin.

MATERIALS AND METHODS

Bacterial strains. Twenty strains of *E. chrysanthemi* (Table 1) were used in this study.

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Table 1. Bacterial strains used

Bacterium	Strain	Isolated from
<i>E. chrysanthemi</i> pv. <i>chrysanthemi</i>	Ku 8601 ^{a)}	Pear
" " "	Ichihara 1-1 ^{b)}	Pear
" " "	E8301 ^{c)}	Chrysanthemum
" " pv. <i>zeae</i>	R7 ^{c)}	Rice
" " "	R8 ^{c)}	Rice
" " "	511-3 ^{a)}	Corn
" " "	Corn 801 ^{c)}	Corn
" " "	Corn 802 ^{c)}	Corn
" " "	Corn 803 ^{c)}	Corn
" " "	NCPPB 377 ^{e)}	Corn
" " "	ALE 8292p ^{c)}	Welsh onion
" " pv. <i>dianthicola</i>	Dianth 2n ^{c)}	Carnation
" " "	Dianth 1e ^{c)}	Carnation
<i>E. chrysanthemi</i> (pv. not identified)	Ech 33 ^{a)}	Taro
" "	Ech 44 ^{a)}	Taro
" "	342-15 ^{a)}	Onion
" "	342-16-S ^{a)}	Onion
" "	329-21 ^{a)}	Mung bean
" "	329-23 ^{a)}	Mung bean
" "	E7188 ^{d)}	Potato
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>	N7129 ^{a)}	Radish

a) Plant Pathology Laboratory, Kyushu University.

b) From Dr. K. Suyama, Tokyo University of Agriculture.

c) From Dr. M. Goto, Shizuoka University.

d) From Dr. K. Tsuchiya, National Institute of Agro-Environmental Sciences, Tsukuba, Ibaraki.

e) Purchased from National Collection of Plant Pathogenic Bacteria (NCPPB), UK.

Each bacterial strain pre-cultured on a slant of YPDA medium (yeast extract 3.0 g, peptone 0.6 g, dextrose 3.0 g, agar 15 g, water 1 liter, pH 7.2) was used for inoculation to 250 ml of liquid 523 medium¹⁰⁾ in 500 ml Sakaguchi flask. Bacteria were grown under shaking condition (156 strokes/min) at 30°C for 24 hr. Cells were harvested by centrifugation at 5,000 × *g* for 30 min at 4°C.

Membrane protein extraction. Membrane proteins were extracted by the method of Frash and Gotschlich⁶⁾. Bacterial cells were washed once with 0.85% NaCl solution by centrifugation and 10 g (f. wt.) of the cells were resuspended in 100 ml of 0.2 M LiCl solution. Seven grams and a half of glass beads (1 mm in diameter) were added to the suspension and agitated (156 strokes/min) at 45°C for 2.5 hr. The suspension was centrifuged at 5,000 × *g* for 20 min at 4°C. The supernatant fluid was recentrifuged at 30,000 × *g* for 40 min to remove large membraneous materials. The resulting supernatant was then centrifuged at 100,000 × *g* at 4°C for 2 hr and the pellet was washed once with distilled water by the same centrifugal condition. The pellet was resuspended in a solution of 0.02% NaN₃ and stored at 4°C until use.

SDS-PAGE. SDS-PAGE was performed in vertical slab gel with Laemmli's discontinuous buffer system¹³⁾. Concentration of acrylamide was adjusted at 5% for stacking and 15% for separating gel. Concentration of protein in each sample was measured by Lowry's folin-phenol method¹⁵⁾. The samples were dissolved in the same volume of double strength of Laemmli's sample buffer¹³⁾ [SDS 0.92 g, 2-mercaptoethanol 2 ml, glycerol 4.0 g, Tris 0.3 g and 0.1% w/v bromophenol blue in 20 ml of total volume] and boiled at 100°C for 3 min. Twenty µl of each dissolved sample was subjected to sample well and electrophoresed at a constant current of 30 mA.

Protein staining and destaining. Proteins were stained overnight with 0.2% co-

omassie brilliant blue R dissolved in the mixed solvent of methanol, glacial acetic acid and water (5:1:4)²¹. The gel was then destained in the mixture of methanol, glacial acetic acid and water (1:1:8). Both staining and destaining were carried out under gentle shaking.

Determination of Rf value and molecular weight. Rf value was calculated by the following formula: $Rf = d/ds$, where d represents the distance from the origin to the sample protein band and ds represents the distance from the origin to the solvent front marked by bromophenol blue²⁰. For molecular weight calculation, phosphorylase b (92.5 kd), bovine serum albumin (BSA) (67.0 kd), egg albumin (45.0 kd), carbonic anhydrase (29.0 kd), trypsin inhibitor from soybean (21.0 kd), cytochrome c (12.5 kd) and trypsin inhibitor from bovine lung (6.5 kd) were simultaneously electrophoresed¹⁸.

Antisera. Three strains of *E. chrysanthemi*, Ku8601 (pear), R8 (rice) and 511-3 (corn), and one strain *E. carotovora* subsp. *carotovora* N7129 (radish) were grown on YPDA medium at 30°C for 24 hr. Bacterial cells were collected from the medium and washed three times with 0.1 M phosphate buffer containing 0.85% NaCl (PBS) by centrifugation at $5,000 \times g$ for 5 min. The washed bacterial cells were resuspended in the saline solution at the concentration of ca. 10^7 cfu/ml and stored at -20°C until use. New Zealand white rabbits were immunized by intravenous injections with 0.3, 0.3, 1.0, 1.0, 1.5 and 1.5 ml of bacterial suspension at 5 days intervals. The blood obtained one week after the final injection was stored for half a day at room temperature, centrifuged at $5,000 \times g$ for 20 min, and the serum fraction was stored at -20°C.

Ouchterlony double diffusion test. The medium containing 0.75% agar noble (Difco) and 0.04% sodium azide in 0.01 M PBS (pH 7.4), was autoclaved at 121°C for 20 min¹⁹. Three ml of the medium was solidified on a slide glass and kept at 4°C until use. Hexagonal well pattern consisting of a central well (4 mm in diameter) surrounded by 6 outer wells with distance of 9 mm each other was prepared and maintained at 30°C for 24 hr before use.

Each 15 ml of the heat-treated (100°C for 20 min) bacterial suspensions (ca. 10^9 cfu/ml) and membrane protein extracts were used as antigens.

Protein blotting. Polypeptide bands separated by SDS-PAGE were transferred from the gel onto a nitrocellulose sheet by vacuum blotting method^{16,17}. The transfer buffer [Tris (25 mM) and glycine (150 mM), pH 8.3] was used as described by Towbin *et al.*²³

Immunodetection. For immunodetection, antisera against *E. chrysanthemi* strains Ku8601, 511-3 and R8 were used as first antibody and anti-rabbit goat IgG conjugated with alkaline phosphatase was used as second antibody¹².

The blotted nitrocellulose sheet was blocked overnight in blocking solution (4% BSA in 0.05 M Tris-HCl, 0.15 M NaCl, 0.5% Tween 20, pH 10.3). The blocked sheet was incubated in the first antibody solution (0.1% BSA in 0.05 M Tris-HCl, 0.15 M NaCl, 0.5% Tween 20, pH 10.3 containing 0.1% of antiserum) for 1 hr. After incubation the sheet was washed (5 min $\times$ 4 times) in phosphate buffered saline with 0.05% Tween 80, incubated in conjugate solution containing 0.05% of the second antibody in the same buffer solution as used for the first antibody solution for 1 hr and washed (5 min $\times$ 4 times) in PBS-Tween. The sheet was immersed in the substrate solution (naphtol ASMX phosphate 0.4 mg/ml in distilled water and Fast red TR salt 6 mg/ml in 0.2 M Tris-HCl, pH 8.2)¹⁹. When color developed sufficiently, the nitrocellulose sheet was photographed through green filter against fluorescent light.

Peptide mapping. *In situ* peptide mapping was carried out as the method developed by Cleveland⁹. The major polypeptides associated with host specificity and antigenicity were cut out from the PAGE gel. The polypeptide samples were partially digested with *Staphylococcus* V8 protease in new PAGE gel and electrophoresed simultaneously. The resolved peptides were detected by silver staining kit (2D-Silver Stain II "Daiichi", Daiichi Pure Chemicals Co., Ltd.).

RESULTS

Ouchterlony double diffusion test

As shown in Table 2, the strains of *E. chrysanthemi* from different hosts showed serologically different reactions where they were divided into four groups (Table 3). Most of the reactions were observed to form one characteristic major precipitin band. The strains that reacted with anti-Ku8601-serum were composed of those isolated from several hosts such as carnation, pear, potato, onion, welsh onion and taro (serogroup I). The strains that reacted only with anti-R8-serum were those isolated from rice (serogroup II). The strains isolated from corn reacted

Table 2. Serological reaction of the whole cells and membrane proteins of *Erwinia chrysanthemi* strains against antisera of Ku8601, R8, 511-3 and *E. carotovora* subsp. *carotovora* N7129, based on precipitin band

Strain (host of origin)	Anti-Ku8601-serum		Anti-R8-serum		Anti-511-3-serum		Anti-Ecc ^{a)} N7129-serum	
	WC ^{b)}	MP ^{c)}	WC	MP	WC	MP	WC	MP
Ku8601 (pear)	+	+	—	—	—	—	—	—
Ichihara 1-1 (pear)	+	+	—	—	—	—	—	—
E8301 (chrysanthemum)	—	—	—	—	—	—	—	—
R7, R8 (rice)	—	—	+	+	—	—	—	—
511-3 (corn)	—	—	—	—	+	+	—	—
Corn 801 (corn)	—	—	—	—	+	+	—	—
Corn 802, 803 (corn)	—	—	—	—	+	+	—	—
NCPB 377 (corn)	—	—	—	—	+	+	—	—
Dianth 2n, 1e (carnation)	+	+	—	—	—	—	—	—
ALE 8292p (welsh onion)	+	+	—	—	—	—	—	—
Ech 33, Ech 44 (taro)	+	+	—	—	—	—	—	—
342-15 (onion)	+	+	—	—	—	—	—	—
342-16-s (onion)	+	+	—	—	—	—	—	—
329-21 (mung bean)	—	—	—	—	—	—	—	—
329-23 (mung bean)	—	—	—	—	—	—	—	—
E7188 (potato)	+	+	—	—	—	—	—	—
E.c.c. ^{a)} , N7129 (radish)	—	—	—	—	—	—	+	+

a) E.c.c. = *E. carotovora* subsp. *carotovora*.

b) WC = Whole cell.

c) MP = Membrane protein.

Table 3. Serogroups of *Erwinia chrysanthemi*

Serogroup ^{a)}			
I	II	III	Undetermined
Ku8601	R7	511-3	329-21
Ichihara 1-1	R8	Corn 801	329-23
Dianth 2n, Dianth 1e		Corn 802	E8301
ALE 8292p		Corn 803	
Ech 33, Ech 44		NCPB 377	
342-15, 342-16-s			
E7188			

a) Serogroup I: positive reaction with anti-Ku8601-serum. II: positive reaction with anti-R8-serum. III: positive reaction with anti-511-3-serum. Undetermined: no reaction with any antisera used.

only with anti-511-3-serum (serogroup III). Thus, anti-R8-serum and anti-511-3-serum were specific for rice strains and corn strains, respectively (Tables 2 and 3). No detectable differences were observed in the reactions against these antisera between heat-treated whole cells and membrane proteins used as antigens (Table 2).

Antigen determinant

Several polypeptides separated in SDS-PAGE were recognized to be positive in serological reaction. Among serogroup I, diversity in pattern of immunopositive polypeptide was observed (Fig. 1 A1–C1). Some of the membrane proteins (41.7, 39.8, 38.9, 38.5 and 33.3 kD)

Fig. 1. Immunodetection of *Erwinia chrysanthemi* membrane antigenic proteins. A, B and C: SDS-PAGE patterns of Ku8601 (pear), 342-15 (onion) and Dianth 1e (carnation) strains, respectively. A1, B1, and C1: Immunodetected antigenic proteins of the same strains.

Fig. 2. Immunodetection of *Erwinia chrysanthemi* membrane antigenic proteins. A and B: SDS-PAGE patterns of R8 (rice) and 511-3 (corn) strains, respectively. A1 and B1: Immunodetected antigenic proteins of the same strains.

associated with host of origin²⁴⁾ were reacted positively. The bands of 38.5 kd and 5.9 kd were commonly positive in all strains of this serogroup. In the strains of serogroup II, the band of 41.7 kd was associated with host of origin and 11.2 kd reacted positively (Fig. 2 A1). In all strains belonging to serogroup III, the bands of 41.7 and 40.3 kd associated with host of origin and the band of 21.4 kd and 10.2 kd were antigenic (Fig. 2 B1). The strains from chrysanthemum and mung bean did not react with any antisera used.

Fig. 3. Peptide mapping of the host-associated membrane proteins of *Erwinia chrysanthemi* strains isolated from: carnation (CA), potato (PO), and taro (TA). E=V8 protease. MW. in (kd).

Fig. 4. Peptide mapping of the host-associated membrane proteins of *Erwinia chrysanthemi* strains isolated from: pear (PE), welsh onion (WO) and chrysanthemum (CH). E=V8 protease. MW. in (kd).

Fig. 5. Peptide mapping of the host-associated membrane proteins of *Erwinia chrysanthemi* strains isolated from: corn (CO), rice (RI) and mung bean (MB). E=V8 protease. MW. in (kd).

Table 4. Peptide mapping of the host-associated membrane proteins of *Erwinia chrysanthemi* strains

Band	MW in (kd)	Strain from ^{a)}									
		WO	ON	PE	CH	MB	CA	PO	TA	CO	RI
a	(42.2)				a ^{b)}						
b	(41.7)			b1 ⁺			b2 ⁺	b2 ⁺	b3	b4 [°]	b5 [*]
c	(40.7)				c1	c2			c3		
d	(40.3)									d [°]	
e	(40.0)										e
f	(39.8)	f1 ⁺	f1 ⁺	f2							
g	(38.9)				g1	g2					
h	(38.5)	h1 ⁺	h1 ⁺	h1 ⁺		h2	h3 ⁺	h3 ⁺	h4 ⁺	h4	h4

a) WO=welsh onion, ON=onion, PE=pear, CH=chrysanthemum, MB=mung bean, CA=carnation, PO= potato, TA=taro, CO=corn, RI=rice.

b) The same symbols represent the same peptide pattern. +, *, ° = antigenic proteins contained in serogroup I, II and III, respectively.

Peptide mapping

As shown in Fig. 3, Fig. 4 and Table 4, when the polypeptide band of 38.5 kd of all strains in serogroup I was partially digested and electrophoresed again, the same peptide map appeared in the strains from pear, onion and welsh onion. The strains from carnation and potato produced the same peptide composition but different from the above 3 strains. The taro strain was different from all of the other strains in serogroup I. The strains isolated from rice and corn showed different pattern in the peptide maps of polypeptide bands of 41.7, 40.3 and 40.0 kd but similar in the maps of the band of 38.5 kd (Fig. 5 and Table 4).

DISCUSSION

As shown in Table 2, *E. chrysanthemi* strains isolated from various hosts are serologically different, supporting the results reported by many workers^{1,3,4,14,25).}

In our study we used 4 types of antisera viz. those against *E. chrysanthemi* strains Ku8601 (pear), R8 (rice) and 511-3 (corn) and that against *E. carotovora* subsp. *carotovora* N7129. The anti-Ku8601-serum reacted with many strains from various hosts such as carnation, pear, potato, onion, taro and welsh onion showing no relationship to origins of these strains. Anti-R8-serum and anti-511-3-serum reacted strongly with those isolated from rice and corn, respectively, suggesting close relationship between serological reaction and host of origin, in contrast to those reported by Janse and Ruissen⁹⁾ that used somatic (O) and flagellar (H) antigens. No detectable differences were observed in antigenicity between heat-treated whole cells and membrane proteins indicating that the heat stable antigen determinants were located mainly in the membrane proteins.

It has been suggested by Bradshaw-Rouse *et al.*¹⁾ that the sugar component of lipopolysaccharide of *E. chrysanthemi* may be major antigen determinant. Yakrus and Schaad²⁵⁾ suggested that antigen determinants of *E. chrysanthemi* were not proteins or are too high to enter the gel. However, our results of immunoblotting test indicated that several polypeptides of membrane proteins were considered to be important antigen determinants of *E. chrysanthemi*. In addition to this, some of the antigen determinants (5.9, 10.2, 11.2, 21.4, 38.5 and 40.3 kd) detected by immunoblotting test seemed to be related to serogroup specificity (Figs. 1 and 2). The authors previously reported that polypeptide bands of 42.2, 41.7, 40.7, 40.3, 40.0, 39.8, 38.9, 33.3 and 30.2 kd were closely associated with host specificity and 38.5 kd was considered to be specific to *E. chrysanthemi*²⁴⁾. Strains of serogroup I reacted with anti-Ku8601-serum showing different patterns in immuno-positive polypeptides (Fig. 1A-C). Among these polypeptides bands, the bands of 38.5 kd and 5.9 kd were commonly found in all strains of this serogroup. This suggests that polypeptide band of 38.5 kd may play important roles as antigen determinant in serogroup I. By peptide mapping of this 38.5 kd band isolated from all strains in serogroups I, II and III it was confirmed that the pattern of peptides of this band was different showing close relationship with host of origin (Figs. 3 and 4 and Table 4). Strains of serogroup II that reacted with anti-R8-serum were characterized by immuno-positive two bands of 41.7 and 11.2 kd (Fig. 2A1). Peptide of 41.7 kd seemed to be related with host of origin of the bacterium (Table 4). Peptide mapping of 41.7 kd band in the strains of serogroup II showed no differences in constitution (Fig. 5). Strains of serogroup III, reacted with anti-511-3-serum, had immuno-positive bands of 10.2, 21.4, 40.3 and 41.7 kd (Fig. 2 A and A1). Both bands of 40.3 and 41.7 kd, which are considered to be related to host of origin, were similar in peptide constitution in all strains of serogroup III (Fig. 5 and Table 4). These results suggested that some of the specific immuno-positive bands in each group were composed of the same peptides and closely related to serogroup and/or host of origin.

The peptide pattern of the membrane proteins of 41.7 kd specific for rice strains was different from that of corn strains (Fig. 5). Since this band is one of the antigen determinants, the difference in peptide map may result in the difference in serological reaction of whole cell antigens. On the other hand, the peptide map of 38.5 kd from rice and corn strains showed similarity (Fig. 5) but they did not react against the antisera used (Table 4). It might be possible to detect other antigen determinants if minor polypeptides will be focused. As observed in Table 4, the band of 38.5 kd from corn, rice and taro presented same peptide map but only the band from taro strain was antigen determinant. This fact suggests difference in the arrangement of antigenic peptide in the membrane. To clarify this, further studies are needed.

Despite the difference in some biochemical properties and pathogenicity, Goto^{7,8)} considered that the strains from rice and corn are not distinguishable into infrasubspecific taxa. However, in this study both strains from rice and corn were serologically distinct even in the membrane protein pattern²⁴⁾.

The serological properties of *E. chrysanthemi* strains were closely related with their host of origin in the case of the strains isolated from rice and corn. Other strains, however, did not show any relationships between serological properties and host of origin as reported by

many workers^{1,3,4,11}.

The proteins composed of bacterial membrane are considered to be the most important structure in the first step of the host-pathogen interaction which may play an important role in determining the degree of compatibility between host and parasite. Our results suggest that some of these proteins are closely related to the host of origin of *E. chrysanthemi* strains. Further analysis of these proteins may lead us to elucidate the mechanism of recognition of host plants by pathogenic bacteria.

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和 文 摘 要

Carlos Hidemi UESUGI・津野和宣・脇本 哲: *Erwinia chrysanthemi* 菌株の抗原決定基

病原性の異なる *Erwinia chrysanthemi* の3系統, すなわち, Ku8601 (ナシからの分離菌), R8 (イネからの分離菌) および 511-3 (トウモロコシからの分離菌) を用いて抗血清を作製し, これらの抗血清に対する反応によって各種の宿主植物から分離された *E. chrysanthemi* の血清グループを比較した。その結果, 供試した系統は4種類の血清グループに分類された。イネおよびトウモロコシを除く各種の植物からの分離菌は抗-Ku8601 血清のみと反応し, これらを血清グループ I とした。イネおよびトウモロコシから分離した菌株はそれぞれ抗-R8 血清および抗-511-3 血清とのみ反応して病原性との間に密接な関連性を示し, 血清グループ II および III とした。またキクおよびマングビーンから分離した系統はいずれの抗血清とも反応しなかった。これら各菌株の膜蛋白を SDS-ポリアクリルアミドゲル電気泳動で分析し, ニトロセルロースシート法により抗原蛋白を検出した。抗-Ku8601 血清と反応する菌株はすべて 38.5 と 5.9 kd の抗原蛋白を所有し, 抗-R8 血清と反応するものは, 41.7 と 11.2 kd の抗原蛋白をもち, また抗-511-3 血清と反応するものは 41.7, 40.3, 21.4 と 10.2 kd の抗原蛋白をもっていた。これらの結果から, *E. chrysanthemi* の抗原決定基は主に菌体の膜蛋白に存在し, これらの蛋白のあるものは宿主に対する親和性の決定に重要な役割を担っている可能性が推察された。