

Xanthomonas campestris pv. citriのhrp遺伝子群の単離とこれをプローブとして用いたXanthomonas属細菌のRFLP解析

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Isolation of *hrp* Cluster from *Xanthomonas campestris* pv. *citri* and Its Application for RFLP Analyses of Xanthomonads

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Abstract

Two cosmid clones (pXCF13-38 and pXCF13-44) were isolated from a genomic library of *Xanthomonas campestris* pv. *citri* strain L-9 using pVir2, a cosmid clone of the *hrp* cluster of *Ralstonia solanacearum*, as the probe. 25 kb region consisting on these clones was identified as the *hrp* region from a test of pathogenicity on citrus and of the hypersensitive response on tobacco after marker exchange of Tn3-Spice insertions. As pXCF13-38 covered almost the entire *hrp* cluster, this clone was used as the probe for RFLP analyses of xanthomonads. A dendrogram was drawn based on analyses of RFLP patterns and was distinguishable from reported dendrograms based on biochemical characters and on RFLP analyses using randomly chosen probes. From these results, the usefulness of RFLP analysis of *hrp* clusters for identification of various xanthomonads was shown and independent evolution of *hrp* cluster in xanthomonads was suggested.

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Key words: *hrp* cluster, *Xanthomonas campestris* pv. *citri*, RFLP.

INTRODUCTION

The detection and identification of phytopathogenic bacteria have been based mostly on biochemical, serological and pathological tests. Recently, chemical methods such as fatty acid²¹⁾ analysis and analysis of DNA homology for particular gene(s) have been widely utilized^{5,11)}. Although the analysis of DNA homology is rapid and requires relatively easy techniques, it is important to choose appropriate gene(s) to examine.

The *hrp* gene of plant pathogenic bacteria, which was first identified in *Pseudomonas syringae* pv. *phaseolicola*, has been reported to be responsible both for disease development on the host plant and for the induction of a hypersensitive reaction (HR) on non-host plants¹⁶⁾. The *hrp* genes isolated from many plant pathogenic bacteria are clustered in a large DNA segment consisting of 20 to 30 kb⁶⁾ except in the case of *hrpM*¹⁷⁾. The *hrp* cluster of plant pathogenic bacteria has been shown to be highly conserved within the same group of bacteria. Thus, the *hrp* cluster was considered to be a useful probe for the detection and identification of phytopathogenic bacteria using RFLP analysis.

Here, we have isolated and characterized the *hrp* cluster of *Xanthomonas campestris* pv. *citri*, and we have confirmed the presence of homologous region in various

xanthomonads. When RFLP patterns in pathovars of *Xanthomonas campestris* using this *hrp* cluster were compared, all of the tested pathovars showed unique patterns: the possible application for the rapid identification of pathovars of *Xanthomonas campestris* will be discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids and media Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured in Luria-Bertani (LB) medium (0.5% yeast extract, 1% peptone, 1% NaCl, pH 7.0) at 37°C. The strains of xanthomonads were cultured in Yeast-Peptone (YP) medium (0.5% yeast extract, 1% peptone, pH 6.8) at 27°C. For solid medium, 1.5% agar was added. The final concentrations of the antibiotics incorporated into these media were 75 µg/ml for kanamycin (Km), 50 µg/ml for nalidixic acid (Nal), 50 µg/ml spectinomycin (Sp) and 25 µg/ml for tetracycline (Tet).

DNA manipulations DNA manipulations were done as described by Maniatis *et al.*¹⁸⁾ and Ausbel *et al.*⁴⁾. *In vitro* λ packaging and purification of DNA fragments from agarose gels were performed by the methods described by the manufactures (Stratagene and Qiagen). Hybridization was performed for 12-18 hr at

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Table 1. Bacterial strains and plasmids used in this study

Bacteria	Description
<i>Xanthomonas campestris</i>	
pv. <i>alfalfae</i>	MAFF03-01090
pv. <i>begoniae</i>	Xbeg8101
pv. <i>campestris</i>	XC8712, XCC4
pv. <i>cannabis</i>	MAFF03-01606, MAFF03-01607
pv. <i>carotae</i>	MAFF03-01101, MAFF03-01111
pv. <i>citri</i>	citrus802, L3, L-9, N1, NA-1, OM, #2-1, #2-2, IV-1, T-1,
pv. <i>glycines</i>	TMR196, TMR200, TMR201, TMR496
pv. <i>physalidicola</i>	MAFF03-01206
pv. <i>pruni</i>	MAFF03-01421, MAFF03-01424, XPR8901
pv. <i>vesicatoria</i>	MAFF03-01256, MAFF03-01262, HJ39 (Isolated from pepper), TOMXV8901 (Isolated from tomato)
pv. <i>vitians</i>	lettuce 80-1, MAFF03-01352, MAFF03-01771
pv. <i>zantedeschiae</i>	MAFF03-01639, MAFF03-01640
<i>Xanthomonas oryzae</i>	
pv. <i>oryzae</i>	T7147 (race2), H75373 (race4)
<i>Escherichia coli</i>	
DH5 α	<i>F</i> ⁻ , <i>endA1</i> , <i>hsdR17</i> (<i>rk</i> ⁺ <i>mk</i> ⁻), <i>supE44</i> , <i>thi-1</i> , <i>recA1</i> , <i>gyrA96</i> , <i>relA1</i> , ϕ 80 <i>dlacZ</i> Δ <i>M15</i> Δ (<i>lacZYA-argF</i>)U169
Plasmids	Description
pVir2	<i>Ralstonia solanacearum</i> DNA cloned in pLAFR3 (Boucher 1987 ⁸⁾)
pXCF13-38	24.7-kb fragment of <i>hrp</i> homologous region cloned in pLAFR5, Tet ^r
pXCF13-44	25-kb fragment of <i>hrp</i> homologous region cloned in pLAFR5, Tet ^r
pLAFR5	<i>IncP</i> , <i>mob</i> , Tet ^r , cosmid vector
pRK2013	<i>IncP</i> , <i>Tra</i> , RK2 ⁺ , Δ <i>repRK2</i> , <i>repE1</i> ⁺ , Km ^r helper plasmid used for triparental mating
Tn3-Spice	<i>inaZ</i> , Amp ^r , Sp ^r , Sm ^r (Lindgren 1987 ¹⁵⁾)
pShee	<i>tnpA</i> , pACYC replicon, Cm ^r (Lindgren 1987 ¹⁵⁾)

The strains named MAFF were from the Genetic Resource Center of the Ministry of Agriculture, Forestry and Fisheries.

65°C in an oven with rotations as described by Maniatis *et al.*¹⁸⁾. The clones of the *hrp* cluster of *Ralstonia solanacearum* and of *X. c.* pv. *citri* were labeled with ³²P-dCTP and used as the probes for hybridization experiments. The blots were exposed to Hyperfilm-MP (Amersham) in a cassette with X-ray intensifying screen.

Construction of a genomic library Total DNA from NA-1 strain of *X. c.* pv. *citri* was partially digested with *Sau3A*I. Treated DNA was fractionated by sucrose gradient to obtain fragments larger than 25 kb, treated with alkaline phosphatase, then ligated at *Bam*HI site on cosmid vector pLAFR5¹³⁾. The ligated DNA was packaged with Gigapack Plus Packaging Kit (Stratagene). The packaged phage particles were used to infect *E. coli* DH5 α . The transformants were selected by their resistance to Tet and their white colony color on X-gal plate.

Marker-exchange mutagenesis in strain NA-1 The mutants by insertion with Tn3-Spice in *hrp* cluster on pXCF13-38 were isolated as described previously¹⁵⁾. Triparental mating was carried out by placing a mixture of the suspensions of *X. c.* pv. *citri*, *E. coli* DH5 α (pXCF13-38::Tn3-Spice) and helper strain *E. coli* HB101 (pRK2013) at a ratio of 10:1:1 on a nitrocellulose membrane (pore size 0.2 μ m, Toyo Roshi Co., Ltd.) placed on YP agar and incubated at 28°C overnight. This mixture of bacterial cells grown on the filter was

suspended in 1 ml of sterilized distilled water, and 100 μ l of the suspension was spread on YP supplemented with NaI, Sp and Tet. Marker-exchanged mutants in the *hrp* cluster were verified by sensitivity to Tet and by Southern analysis using Tn3-Spice as the probe.

Inoculation tests The seeds of *Citrus natsudaidai* and the seeds of *Nicotiana tabacum* were planted in 15 cm-diameter pots and cultivated in a growth chamber for longer than 14 days followed by cultivation in greenhouse. The suspension of bacteria (1.0×10^8 cfu/ml) in sterile distilled water was injected into the intercellular space of leaves on 2-month-old seedlings. After inoculation, plants were incubated in a growth chamber at 27°C, and symptom appearance was checked periodically.

RFLP analysis In this study, only the hybridized bands larger than 1 kb, regardless of their intensity, were compared. The bands of each strain were recorded as a binary file by scoring for the presence or absence of each band. Dice's coefficient of similarity was computed from the binary data by using the Qualitative program in NTSYS-PC version 1.80¹⁹⁾. Then, a dendrogram was constructed from the distance matrix data by using the unweighted pair group method with averages (UPGMA) with the NEIGHBOR program in PHYLIP package¹⁰⁾.

RESULTS

Isolation of cosmid clone containing *hrp* homologous regions

Cosmid library of *X. c. pv. citri* strain L-9 was constructed in *E. coli* DH5 α using pLAFR5 as the vector. From restriction enzyme analysis of 30 randomly selected colonies, the average size of the inserts was estimated to be about 25 kb (data not shown). Two cosmids pXCF13-38 and pXCF13-44 out of 2100 clones were found to contain the region homologous to pVir2, a clone containing the entire *hrp* cluster of *Ralstonia solanacearum* strain GMI1000⁽³⁾ (Fig. 1). The *Eco*RI fragments of *X. c. pv. citri* homologous to the corresponding fragments of the *hrp* cluster of *R. solanacearum* were summarized in Fig. 1. Southern analyses of *Eco*RI digests of two cosmid clones using pVir2 as the probe indicated that fragments homologous to pVir2 were

found in a continuous region of about 14 kb. Three tandem *Eco*RI fragments (total of 17 kb) located at the 5' part of pVir2 hybridized with this 14 kb (Fig. 1). In other words, a 25-kb *hrp* cluster of *X. c. pv. citri* contains both 14 kb of a well-conserved region even among different genera and 10 kb of a less conserved region.

Tn3 mutagenesis of *hrp* cluster of *X. c. pv. citri*

Fifty strains randomly inserted with Tn3-Spice into pXCF13-38 and pXCF13-44 were isolated. From analysis of ice-nucleation activity, forty-six insertions were shown to be inserted in the direction of the transcription of the genes on these cosmid clones. The marker-exchanged strains in *X. c. pv. citri* using these inserted constructs were tested for pathogenicity on citrus seedlings and for HR-inducing activity on tobacco plants (Fig. 2). Insertions into the region of the 25-kb fragment resulted in the loss of both pathogenicity and HR-inducing ability. Insertions in another region of about 25 kb resulted in the loss of either pathogenicity or HR-

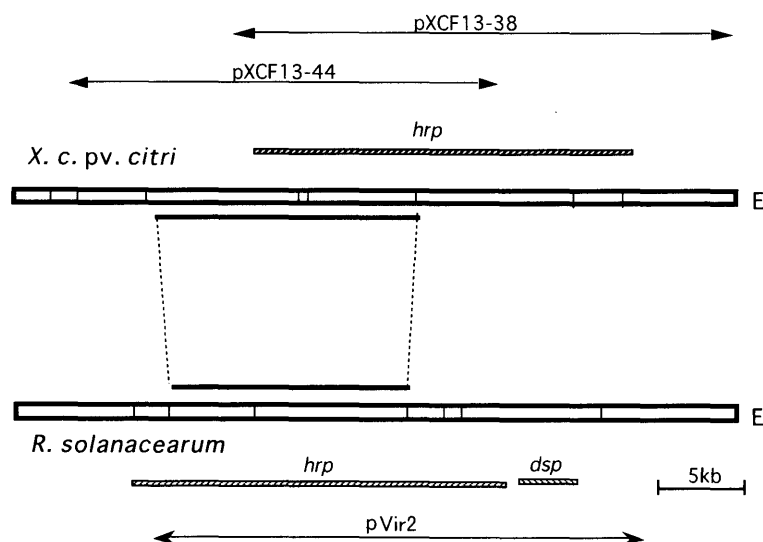


Fig. 1. Structural homology between the region of *Ralstonia solanacearum* and corresponding region of *Xanthomonas campestris* pv. *citri*. The homologous *Eco*RI fragments between pXCF13-38, pXCF13-44 and pVir2 are shown by thick bars. The hatched bars represent the *hrp* gene clusters of *R. solanacearum* and *X. c. pv. citri*. Abbreviation: E, *Eco*RI.

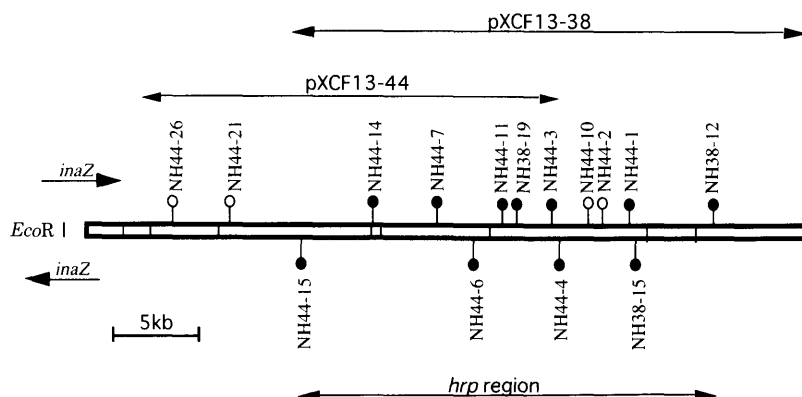


Fig. 2. Mutation analysis of the *hrp* *X. c. pv. citri*. Filled and open circles at the restriction map indicate the position of Tn3-Spice insertion mutants which were complemented and not complemented with pXCF13-38, respectively. The arrows indicate the direction of transcription determined by ice-nucleation activity.

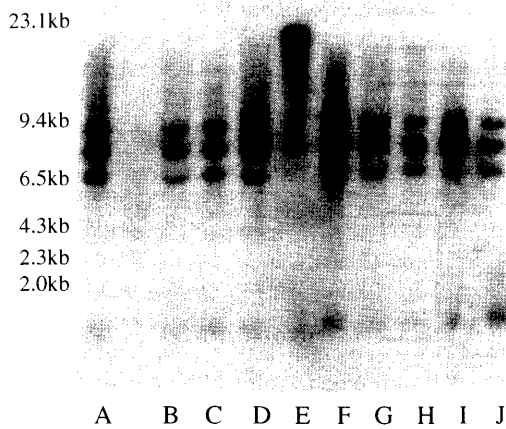


Fig. 3. Hybridization of the clone of *hrp* cluster (pXCF13-38) to total DNA of strains *X. c. pv. citri*. Approximately 10 μ g of *Eco*RI-digested DNA was loaded per lane. The blots were probed with the 32 P-labelled pXCF13-38. A; L-9, B; T-1, C; IV-1, D; #2-2, E; #2-1, F; OM, G; NA-1, H; N1, I; L3, J; citrus 802.

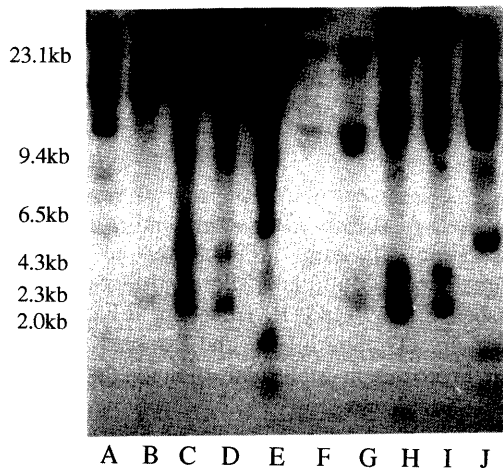
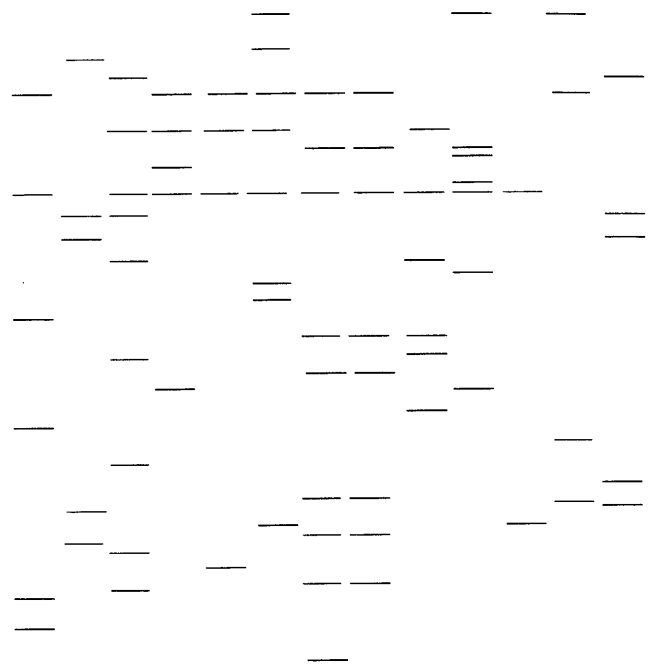


Fig. 4. RFLP of total DNA of strains of *X. c.* pathogens using pXCF13-38 as the probe. Approximately 10 μ g of *Eco*RI-digested DNA was loaded per lane. The blots were probed with the 32 P-labelled pXCF13-38. A; *X. c. pv. physaridicola*, B; *X. c. pv. vesicatoria*, C and D; *X. c. pv. vitians*, E; *X. c. pv. cannabis*, F and G; *X. c. pv. zantedeschiae*, H and I; *X. c. pv. campestris*, J; *X. c. pv. alfalfae*.

inducing activity (Fig. 2). As pXCF13-38 was found to cover entire regions responsible for the *hrp* phenotype, this clone was used as the probe for RFLP analysis.

RFLP analyses of various *Xanthomonas* pathogens

In the case of *X. c. pv. citri*, all ten tested isolates showed identical RFLP patterns (Fig. 3). However, most of the patterns for other pathogens were distinguishable from that of *X. c. pv. citri* (Fig. 4). RFLP patterns of all the tested pathogens were summarized (Fig. 5), and a dendrogram was depicted based on the analysis of these



Alf Cam Can Car Cit Gly Ory II Ory IV Phy Pru Ves Vit Zan

Fig. 5. RFLP of total DNA of strains of *X. c.* pathogens using pXCF13-38 as the probe. This figure was made by tracing RFLP pattern of strains tested in this study. Alf; *X. c. pv. alfalfae*, Cam; *X. c. pv. campestris*, Can; *X. c. pv. cannabis*, Car; *X. c. pv. carotae*, Cit; *X. c. pv. citri*, Gly; *X. c. pv. glycines*, Ory II; *X. o. pv. oryzae* race II, Ory IV; *X. o. pv. oryzae* race IV, Phy *X. c. pv. physaridicola*, Pru; *X. c. pv. pruni*, Ves; *X. c. pv. vesicatoria*, Vit; *X. c. pv. vitians*, Zan; *X. c. pv. zantedeschiae*.

results (Fig. 6). However, unlike the case of *X. c. pv. citri*, the degree of homogeneity in the pattern remains to be studied for other pathogens.

DISCUSSION

The length of the *hrp* cluster of *X. c. pv. citri* was determined to be about 25 kb which is similar to the reported sizes of the *hrp* cluster of *X. c. pv. vesicatoria* and *X. c. pv. campestris*^{2,7)}. The total length of the *hrp* cluster of *X. c. pv. citri* was also similar to that of *R. solanacearum*. However, only 14 kb at the 5' end of the *hrp* cluster of *X. c. pv. citri* hybridized with the *hrp* cluster of *R. solanacearum* (Fig. 1). This region of *R. solanacearum* has been reported to contain many *hrc* genes¹⁴⁾ which are involved in type III secretion^{1,9,12)} and may exist beyond genus and species. On the other hand, the remaining 10-kb region seems to be unique in each genus.

The dendrogram in this study was different from the dendrogram in other studies based on biochemical characters²⁰⁾ and on RFLP analysis using random probes¹¹⁾. This result may indicate that the *hrp* cluster

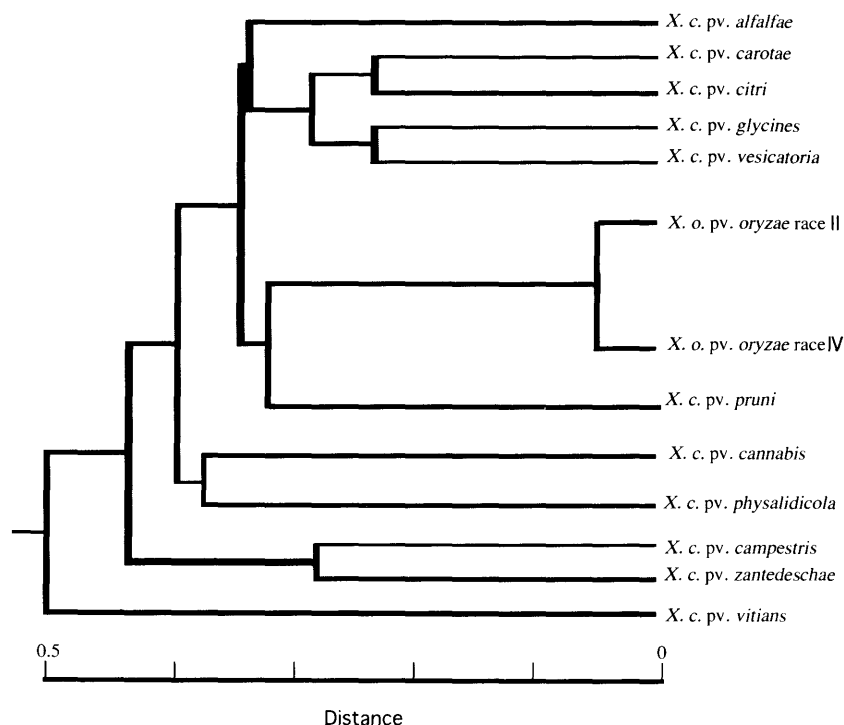


Fig. 6. Dendrogram based on analysis of RFLP patterns of *X. c.* pathovars. Similarity based on scoring the presence or absence of each band was computed by using the Qualitative program in NTSYS-PC version 1.80. A dendrogram was constructed from the distance matrix data by using the unweighted pair group method with averages (UPGMA) with the NEIGHBOR program in PHYLIP package.

may evolve independently from other housekeeping genes of xanthomonads. This aspect needs further RFLP analyses using other pathogenic genes.

Since RFLP patterns using pXCF13-38 as the probe for each *X. c.* pathovar were shown to be distinguishable, this RFLP analysis may be applicable for quick identification of *X. c.* pathovars. At least for *X. c.* pv. *citri*, such identification may be possible. However, to identify other pathovars, homogeneity among many isolates of the pathovar has to be tested.

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和 文 摘 要

金森裕之・杉本仁志・落合弘和・加来久敏・露無慎二： *Xanthomonas campestris* pv. *citri* の *hrp* 遺伝子群の単離とこれをプローブとして用いた *Xanthomonas* 属細菌の RFLP 解析

ナス科植物青枯れ病菌の *hrp* 領域のクローンである pVir2 をプローブとしてカンキツかいよう病菌よりコスミドクローン pXCF13-38 および pXCF13-44 を分離した。これらクローンをを用いてマーカーエクステンジ法により Tn3 を挿入したところ、約 25 kb の領域がカンキツ葉での病原性とタバコでの HR 誘導の双方を司る *hrp* 領域であることが判明した。特に pXCF13-38 は *hrp* 全領域を保持していたため、これを用いて *Xanthomonas* 属細菌の RFLP 解析を行った。その結果、*X. campestris* の各 pathovar を明確に識別できた。また RFLP 解析の結果を基に樹形図を作成したところ、生化学的性状に基づいた解析やランダムプローブを用いた RFLP 解析の樹形図と異なる結果となった。以上のことより、*hrp* 領域を用いた RFLP 解析が *X. campestris* の pathovar 間の識別に有用であることを示すと同時に、*hrp* クラスターが培養学的性状などを司る他の領域とは独立して進化したことを示唆することができた。