J. Microbiol. Biotechnol. (2007), **17**(2), 359–363

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Interaction Proteome Analysis of *Xanthomonas* Hrp Proteins

JANG, MI[1], BYOUNG CHUL PARK[1], DO HEE LEE[1], KWANG-HEE BAE[1], SAYEON CHO[2], HYUN SEOK PARK[3], BAEK RAK LEE[4], AND SUNG GOO PARK[1]*

[1]*Proteome Research Laboratory, KRIBB, Daejeon 305-333, Korea*
[2]*College of Pharmacy, Chung-Ang University, Seoul, Korea*
[3]*Department of Computer Science and Engineering, Ewha Womans University, Seoul, Korea*
[4]*Department of Microbiology, Inje University, Kimhae, Korea*

Received: June 28, 2006
Accepted: August 25, 2006

Abstract Because of the importance of the type III protein-secretion system in bacteria-plant interaction, its function in bacterial pathogenesis of plants has been intensively studied. To identify bacterial proteins interacting with *Xanthomonas hrp* gene products that are involved in pathogenicity, we performed the glutathione-bead binding analysis of *Xanthomonas* lysates containing GST-tagged Hrp proteins. Analysis of glutathione-bead bound proteins by 1-DE and MALDI-TOF has demonstrated that Avr proteins, RecA, and several components of the type III secretion system interact with HrpB protein. This proteomic approach could provide a powerful tool in finding interaction partners of Hrp proteins whose roles in host-pathogen interaction need further studies.

Key words: *hrp* gene, interaction protein, 1-DE, MALDI-TOF, pathogenesis

Numerous microorganisms inhabit plants. For example, diverse endophytic bacteria grow in the vascular system and within the intercellular spaces of plants cells. Although most of these plant-associated bacteria are either symbiotic or commensal with plants, a handful of them have evolved the ability to exploit the plants to gain nutrients and cause diseases in the process [1]. These selected bacteria include proteobacterial genera and Gram-positive genera. Bacterial protein-secretion systems play key roles in the ability of a pathogen to infect a plant. These include proteases secreted by a type I protein-secretion system, a variety of plant cell wall-degrading pectic enzymes secreted by a type II secretion system, effector proteins secreted via type III secretion systems, or the T-DNA-protein complex that is delivered into plant cells via a type IV secretion system. Because of the importance of the type III protein-secretion system in bacteria-host interactions and pathogenesis, this protein-secretion system has been intensively studied. Several Gram-negative plant pathogens use type III protein-secretion systems to deliver bacterial proteins inside the plant cell [11]. The genes encoding type III secretion systems were originally isolated from several bacterial plant pathogens, and these genes have been named as *hrp* genes for hypersensitive response and pathogenicity. The hypersensitive response (HR) is a programmed cell death of plant tissue associated with defense. The *hrp* genes are clustered and usually contained in the chromosome of the pathogen. The *hrp/hrc* cluster of *X. campestris* pv. *vesicatoria* 85-10 is located in the main chromosome [4]. Analysis of DNA adjacent to the *hrp/hrc* cluster in the rice pathogen *X. oryzae* pv. *oryzae (X. o. o.)* identified several genes that are likely to encode type III-secreted proteins [19]. Genes associated with *hrp*, *hpa1*, and *hpa2* are located to the left of the *hrp/hrc* cluster's *hrpA* in *X. o. o.* The *hpa1* gene shares characteristics with PopA and other harpins from several plant pathogens, which appear to act as extracellular helper proteins. The *hpa2* gene encodes a lysozyme-like protein found in type III secretion systems, including that of *P. syringae* and *E. chrysanthemi* [2], as well as other secretion systems in Gram-negative bacteria, and is predicted to act in the periplasm [19]. Proteome analysis is expected to be the bridge between the genomic sequence and proteome. The combination of 1-DE, mass spectrometry (MS) technology, and rapidly accumulating genomic sequence data permits truthful and rapid identification of proteins. With these advances, proteome analysis can be applied to the research of intracellular protein changes in cells following a certain stimulus [3, 5, 6, 12]. To understand the protein network of the type III secretion system and determine their roles in

*Corresponding author
Phone: 82-42-860-4262; Fax: 82-42-860-4269;
E-mail: sgpark@kribb.re.kr

the pathogenesis of plant diseases caused by *Xanthomonas*, we attempted herein to identify *Xanthomonas* proteins interacting with Hrp proteins.

HRP genes - *hrpB1* (*hrpB2*, *hrpB4*, *hrpB5*, *hpa1*, and *hpa2*) were amplified by PCR from genomic DNA of *Xanthomonas oryzae*. Primers used for PCR amplification were as follows; *hrpB1*, CGGGATCC GTG GAG AAG ATT C (forward) and GCGTCGAC TCA GGC GCG CA (reverse); *hrpB2*, CGGGATCC ATG ACG CTC ATT (forward) and GCGTCGAC CTA CTG GTT CTT (reverse); *hrpB4*, CGGGATCC ATG GAC AAC ACG (forward) and GCGTCGAC TCA ACC AGA CAC (reverse); *hrpB5*, CGGGATCC ATG CGT GTC TGG T (forward) and GCGTCGAC TCA GCC AAC ATC C (reverse); *hpa1*, CGGGATCC ATG AAT TCT TTG AA (forward) and GCGTCGAC TTA CTG CAT CGA (reverse); *hpa2*, CGGGATCC GAT TGC TTC GAA TAA (forward) and GCGTCGAC CTA CGC CAC CTG (reverse). The resulting PCR products were cloned into *E. coli* expression vector pGEX6-1 (AmershamPharmacia Biosciences), which was then introduced into BL-21 cells. Transformed BL-21 cells were cultured overnight at 37°C in 2×YT medium supplemented with ampicillin (100 μg/ml), and then transferred to fresh medium to the ratio of 1 to 200. GST-fusion proteins were induced by 1 mM IPTG and purified by using glutathione (GSH)-Sepharose 4B beads (obtained from Peptron Inc.).

Xanthomonas oryzae were initially cultivated in NYG broth overnight at 28°C. The cells were collected by centrifugation at 5,000 rpm for 30 min at 4°C and then cultured again in *hrp*-inducing XVM2 medium [20 mM NaCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4, 0.01 mM $FeSO_4$, 10 mM fructose, 10 mM sucrose, 0.03% casamino acid (pH 6.7)] for 12 h at 28°C. The cells were collected again by centrifugation and washed once with PBST (phosphate-buffered saline containing 1% Trion X-100) to remove culture media. Collected cells were resuspended in PBS and disrupted by sonication (Sanyo Soniprep 150; 10×2 s bursts at amplitude 7.5 min). Cell debris was removed by centrifugation at 13,000 rpm for 30 min at 4°C. GST-fusion proteins were immobilized onto GSH-Sepharose beads by incubating the purified proteins with prewashed beads. As a control, purified GST was adsorbed to beads. After incubation, the beads were washed four times with 100 volumes of PBST, resuspended in an equal volume of PBST, and then stored at 4°C until further use. Next,

Table 1. *Xanthomonas* proteins interacting with HrpB and Hpa proteins.

#	Accession no.	Species[a]	Proteins identification	*pI*[b]	Mw[c] (Da)	MOWSE score	Coverage[d]
	HrpB1						
1	P29956	XANCP	Xanthan biosynthesis protein	5.5	50962	1.82	9
2	Q8PAL1	XANCP	Heat-inducible transcription repressor hrcA	5.9	38290	1.84	6
3	O30633	XANOR	RecA protein (Recombinase A)	5.4	37217	3.26	8
	HrpB2						
4	Q8PP22	XANCV	Organic solvent tolerance protein precursor	5.2	92243	10.3	8
5	Q8PAK9	XANCP	Chaperone protein dnaK	5.0	68838	1.19	4
6	P19520	XANCV	Hypothetical 50 kDa avirulence protein in AVRBS1 region	6.5	49800	321	16
7	Q08678	XANCV	Avirulence protein avrRxv	9.7	42021	9.45	12
8	P80153	XANCV	Probable ATP synthase hrpB6	5.0	47710	20.6	26
9	P25438	XANCV	Insertion element IS476 hypothetical 39.2 kDa protein	10.2	39236	5.91	12
10	O30633	XANOR	RecA protein (Recombinase A)	5.4	37217	1.41	11
	HrpB4						
11	P80153	XANCV	Probable ATP synthase hrpB6	5.0	47710	5.52	9
	HrpB5						
12	P52311	XANOR	Modification methylase XorII	9.6	46971	19.4	4
13	Q8PHZ5	XANAC	Glutathione synthetase (GSH synthetase)	5.8	34322	1.3	11
	Hpa2						
14	P14727	XANCV	Hypothetical 122 kDa avirulence protein in AVRBS3 region	7.5	122278	0.48	3
15	O30633 Hpa1	XANOR	RecA protein (Recombinase A)	5.4	37217	1.34	11
16	P80151	XANCV	Hypersensitivity response secretion protein hrpA1 (Precursor)	5.9	63936	0.78	3
17	Q8PHZ5	XANAC	Glutathione synthetase (GSH synthetase)	5.8	34322/5.8	1.2	24

[a]The species names follow; XANAC, *Xanthomonas axonopodis* (pv. *citri*), XANCP, *Xanthomonas campestris* (pv. *campestris*), XANCV, *Xanthomonas campestris* (pv. *vesicatoria*), XANOR, *Xanthomonas oryzae* (pv. *oryzae*).

[b,c]Theoretical value using ExPASY tool.

[d]Percent coverage obtained for the sequence of the identified protein.

Xanthomonas lysates were incubated with GST or GST-fusion protein immobilized GSH-beads at 4°C for 2 h. The beads were washed four times with 100 bed volumes of PBST buffer to remove nonspecifically bound proteins. The bound proteins were finally eluted from the beads by 1× SDS sample buffer and separated by 1-D SDS-PAGE. Silver staining kit (AmershamPharmacia Bioscience) was used for the visualization of proteins. The target proteins were excised from the gel. Proteins were destained, in gel digested with trypsin. The extracted peptides were put through desalting/concentration step on μZipTipC18 (Millipore Co.) before MALDI-TOF mass spectrometry analysis. Molecular weight information about the peptide was obtained in positive reflected mode in a Voyager-DE PRO mass spectrometer (Applied Biosystems) and external mass calibration using calmix2 (Applied Biosystems). Mass mapping spectra were recorded using α-cyano-4-hydroxycinnamic acid (Sigma) as a matrix (8 mg/ml, H0/CAN/TFA 50:50:0.1). The peptide MS fingerprint was subjected to MS-Fit software searching against the Swiss-Prot database.

In the case of spot8 from HrpB2-interacting proteins, MS/MS analysis was performed to verify the results of MALDI-TOF analysis. For the MS/MS analysis, peptide mass spectra were obtained in positive-ion mode in a MALDI-TOF/TOF mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems). Protein identification was processed and analyzed by searching the NCBI protein database using the MASCOT search engine of Matrix Science that integrated in the Global Protein Server (GPS) Workstation. The mass tolerance was limited to 0.1 Da. Fig. 2 shows that the MS/MS analysis of spot 8 really indicated a HrpB6 protein.

In this study, we isolated a number of GSH bead-bound proteins and identified about 17 proteins that were presumed to specifically interact with Hrp proteins (Table 1 and Fig. 1). Interestingly, some of the Hrp-interacting proteins were shown to associate simultaneously with multiple members of Hrp proteins, suggesting that they might function as nodal proteins in the protein-interaction network of bacterial pathogenesis. Most notably, RecA protein was identified as an interaction partner of HrpB1, HrpB2, and Hpa2. When pathogenic bacteria are exposed to oxidants during infection, they must overcome this damage in order to proliferate in the host plant, and the oxidant attack has been shown to induce RecA in several bacteria [9]. Moreover, the presence of repeated DNA, repeated motifs in avirulence and pathogenicity genes, coupled with an active recombination system, contribute to variation in pathogenicity of bacteria [13]. Furthermore, RecA in *Erwinia* is involved in the regulation of pectinlyase production and pathogenicity [14]. Therefore, RecA may control the pathogenicity of *Xanthomonas* by interacting with multiple Hrp proteins.

The 39-kDa protein in IS476, identified as an interacting partner of HrpB2, has a significant homology with the putative transposase of IS3 from *E. coli*. The position of IS476 insertion in several avrBs1 mutations was shown to influence both induction of hypersensitivity and bacterial growth in plants [10]. Previously, HrpB2 was shown to be secreted via type III secretion systems and is involved in pathogenicity of Avr proteins, and HrpB4 was characterized as an associated component of the type III secretion system [17]. Interaction of HrpB1 with xanthan biosynthesis protein may suggest the role of xanthan production in virulence. In fact, mutants of *X. c. c.* with defects in xanthan production are known to have severely reduced virulence in plants [15]. One possible role of xanthan in the disease process is

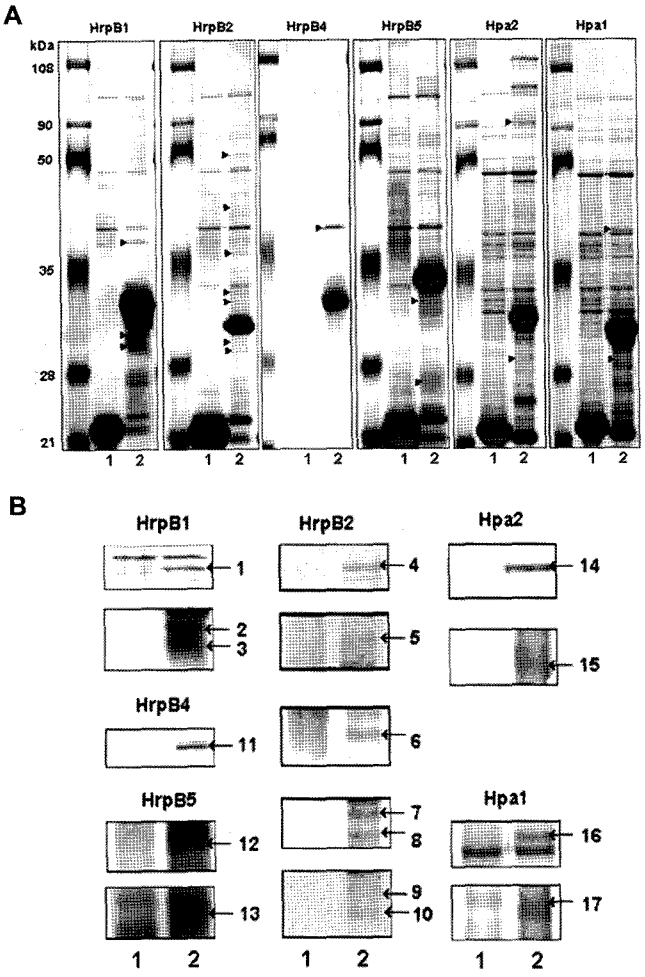

Fig. 1. SDS-PAGE analysis of *Xanthomonas* proteins interacting with Hrp proteins.

A. 1-D gel images of proteins bound to GSH-beads. *Xanthomonas* lysates were applied to GSH-beads pre-absorbed with GST-tagged Hrp or Hpa proteins. After washing with buffer, the proteins bound to beads were eluted with 1× SDS-PAGE sampling buffer and then separated by 10% SDS-PAGE. Specifically bound proteins are indicated by arrowheads. **B.** Close-up of the areas of 1-D gels shows proteins specifically interacting with GST-tagged Hrp proteins. Only spots identified by MALDI-TOF analysis are numbered. Lane 1, proteins eluted from the beads immobilized with GST (control); lane 2, proteins eluted from the beads immobilized with GST-tagged Hrp or Hpa proteins.

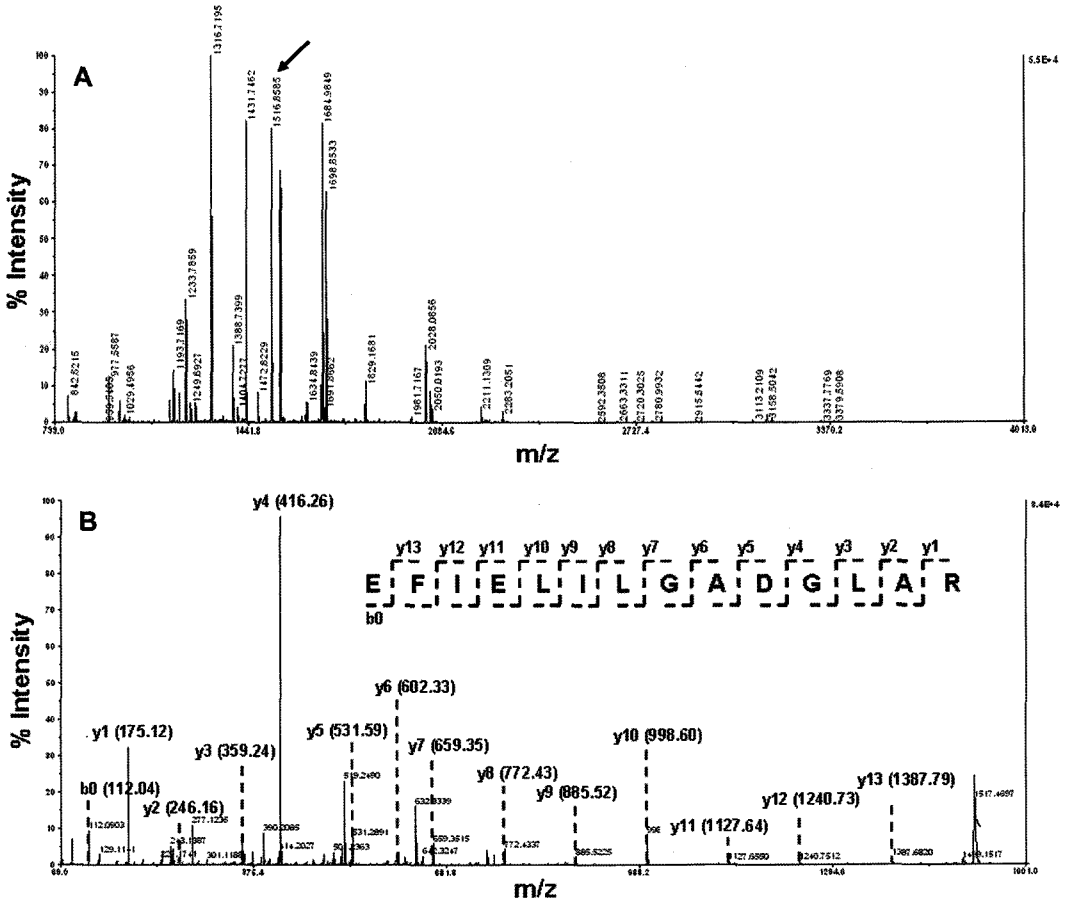

Fig. 2. Analysis of in-gel tryptic digest of the spot 8 by MS (**A**) and MS/MS (**B**).
The MS/MS spectra were used to search the NCBI nonredundant and expressed sequence tag databases using Mascot. Database search was performed with mass tolerance 0.2 Da for MS and 0.1 Da for MS/MS, and with confidence value no less than 80. MS/MS spectra were obtained for the +1 precursor ion with *m/z* of 1516.84 corresponding to a HrpB6 peptide EFIELILGADGLAR.

in the formation of biofilms, which may protect bacteria against stresses such as desiccation and the action of plant derived antimicrobial compounds [7]. HrpB6, interacting with HrpB2 and HrpB4, is known to be necessary for both basic pathogenicity and induction of hypersensitive response in resistant plants. It may function as an ATPase that is related to a transport apparatus rather than as a part of a proton pump [8]. It may also be possible that HrpB6 functions as a component of the type III secretion system for the supply of energy for the transport of harpins.

Two *hrp*-associated loci, named *hpa1* and *hpa2*, were located beyond the *hrpA* operon. A strain with a deletion encompassing *hpa1* and *hpa2* has reduced pathogenicity and elicits a weak HR on non-host and resistant host plants [19]. Hpa1 is a 13-kDa glycine-rich protein with a composition similar to those of harpins and PopA [19], and Hpa2 is similar to lysozyme-like proteins. In this analysis, S-adenosyl-methyltransferase (mraW) and hypothetical 122-kDa avirulence protein in the AVRBS3 region were identified as Hpa1-interacting partners, and hypersensitivity response secretion protein HrpA and glutathione synthetase were screened as Hpa2-interacting partners, respectively (Table 1 and Fig. 1). HrpA1 protein, also a member of Hrp proteins, shows similarity to components of the type II secretion pathway, originally discovered in *Klebsiella oxytoca*, which secretes specific target proteins across the outer membrane to the extracellular milieu [18]. The finding that HrpA1 interacts with Hpa1 suggests that Hpa proteins probably influence the pathogenicity of *Xanthomonas* by affecting the secretion of proteins via the type II secretion system.

Previous studies on plant pathogens have shown that several *hrc* genes, such as *hrcC*, *hrcJ*, *hrcT*, *hrcU*, and *hrcV*, are also required for protein secretion [16]. Moreover, some of the nonconserved *hrp* genes of *X. c.* pv. *vesticatoria* are also required for secretion, and therefore, may encode additional proteins associated with the secretion apparatus [17]. HrpB2 is not a core component of the secretion apparatus, but is crucial for the secretion of other proteins traveling via the type III pathway [17]. The fact that HrpB2 is required for secretion via the Hrp pathway suggests that it is a part of the secretion apparatus. Although it is secreted by the Hrp system, the protein might be localized on the

surface of the bacterium as a part of an appendage such as the Hrp pilus of *P. s.* pv. *tomato* and *R. solanacearum*. This possibility suggests that HrpB2 does not encode auxiliary proteins specific for Avr protein secretion, such as a chaperone or a protein required for recognition of an Avr-specific secretion signal. Avr as well as HrpB2 was secreted by the wild-type strain, but not by any of the *hrpB1*, *hrpB2*, *hrpB4*, and *hrpB5* mutants. Thus, *hrpB1*, *hrpB2*, *hrpB4*, and *hrpB5* are essential for Avr secretion. HrpB1 and HrpB4 were detected mainly in the soluble fraction, whereas HrpB2 was present in both soluble and membrane fractions. Secretion of HrpB2 was abolished in a mutant carrying a deletion in *hrcV*, which encodes a protein conserved in type III secretion systems. Thus, HrpB2 is secreted in a *hrp*-dependent manner.

In addition to the present findings, future studies on localization and protein-protein interaction between Hrp proteins (and between Hpa proteins as well) are needed to accelerate our understanding of the role of individual components of the secretion apparatus in bacterial pathogenicity. Further efforts on elucidating the complete interaction map of Hrp proteins would help find the mechanism of host-ranges, and the frequencies of horizontal transfer of pathogenicity islands and virulence plasmids, as well as detailed analysis of the contribution of each pathogenicity factor to virulence that allowed plant pathogenicity to develop. Moreover, by combining molecular biological techniques with the proteomic technologies, we have an excellent opportunity to make major progresses in understanding the nature of the bacterial pathogenicity of plants with the long-term goal of improving the ability of plants to defend themselves against bacteria.

REFERENCES

1. Alfano, J. R. and A. Collmer. 1996. Bacterial pathogens in plants: Life up against wall. *Plant Cell* **8:** 1683–1698.
2. Alfano, J. R., A. O. Charkowski, W. L. Deng, J. L. Badel, T. Petnicki-Ocwieja, K. van Dijk, and A. Collmer. 2000. The *Pseudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector *loci* that contribute to parasitic fitness and pathogenicity in plants. *Proc. Natl. Acad. Sci. USA* **97:** 4856–4861.
3. Anderson, N. L. and N. G. Anderson. 1998. Proteome and proteomics: New technologies, new concepts, and new words. *Electrophoresis* **19:** 1853–1861.
4. Bonas, U. 1994. *hrp* Genes of phytopathogenic bacteria. *Curr. Top. Microbiol. Immunol.* **192:** 79–98.
5. Choi, N. S., K. H. Yoo, J. H. Hahm, K. S. Yoon, K. T. Chang, B. H. Hyun, P. J. Maeng, and S. H. Kim. 2005. Purification and characterization of a new peptidase, bacillopeptidase DJ-2, having fibrinolytic activity produced by *Bacillus* sp. DJ-2 from Doen-jang. *J. Microbiol. Biotechnol.* **15:** 72–79.
6. Choi, N. S., S. K. Ju, T. Y. Lee, K. S. Yoon, K. T. Chang, P. J. Maeng, and S. H. Kim. 2005. Miniscale identification and characterization of subtilisins from *Bacillus* sp. strains. *J. Microbiol. Biotechnol.* **15:** 537–543.
7. Dow, J. M., L. Crossman, K. Findlay, Y. Q. He, J. X. Feng, and J. L. Tang. 2003. Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. *Proc. Natl. Acad. Sci. USA* **100:** 10995–11000.
8. Fenselan, S., I. Balbo, and U. Bonas. 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. *Mol. Plant Microbe Interact.* **5:** 390–396.
9. Karlin, S., G. M. Weinstock, and V. Brendel. 1995. Bacterial classifications derived from recA protein sequence comparisons. *J. Bacteriol.* **177:** 6881–6893.
10. Kearney, B. and B. J. Staskawicz. 1990. Characterization of IS476 and its role in bacterial spot disease of tomato and pepper. *J. Bacteriol.* **172:** 143–148.
11. Kim, J. F. and J. R. Alfano. 2002. Pathogenicity islands and virulence plasmids of bacterial plant pathogens. *Curr. Top. Microbiol. Immunol.* **264:** 127–147.
12. Kim, M. J., H. J. Chung, S. M. Park, S. G. Park, D. K. Chung, M. S. Yang, and D. H. Kim. 2004. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF)-based cloning of enolase, ENO1, from *Cryphonectria parasitica*. *J. Microbiol. Biotechnol.* **14:** 620–627.
13. Leach, J. E., M. L. Rhoads, C. M. Vera Cruz, F. F. White, T. W. Mew, and H. Leung. 1992. Assessment of genetic diversity and population structure of *Xanthomonas oryzae* pv. *oryzae* with a repetitive DNA element. *Appl. Environ. Microbiol.* **58:** 2188–2195.
14. Liu, Y., A. Chatterjee, and A. K. Chatterjee. 1994. Nucleotide sequence, organization and expression of *rdgA* and *rdgB* genes that regulate pectin lyase production in the plant pathogenic bacterium *Erwinia carotovora* subsp. *carotovora* in response to DNA-damaging agents. *Mol. Microbiol.* **14:** 999–1010.
15. Newman, M. A., J. Conrads-Strauch, G. Scofield, M. J. Daniels, and J. M. Dow. 1994. Defense-related gene induction in *Brassica campestris* in response to defined mutants of *Xanthomonas campestris* with altered pathogenicity. *Mol. Plant Microbe Interact.* **7:** 553–563.
16. Rossier. O., K. Wengelnik, K. Hahn, and U. Bonas. 1999. The *Xanthomonas* Hrp type III system secretes proteins from plant and mammalian bacterial pathogens. *Proc. Natl. Acad. Sci. USA* **96:** 9368–9373.
17. Rossier, O., G. Van den Ackerveken, and U. Bonas. 2000. HrpB2 and HrpF from *Xanthomonas* are type III-secreted proteins and essential for pathogenicity and recognition by the host plant. *Mol. Microbiol.* **38:** 828–838.
18. Wengelnik, K., C. Marie, M. Russel, and U. Bonas. 1996. Expression and localization of HrpA1, a protein of *Xanthomonas campestris* pv. *vesicatoria* essential for pathogenicity and induction of the hypersensitive reaction. *J. Bacteriol.* **178:** 1061–1069.
19. Zhu, W., M. M. MaGbanua, and F. F. White. 2000. Identification of two novel *hrp*-associated genes in the *hrp* gene cluster of *Xanthomonas oryzae* pv. *oryzae*. *J. Bacteriol.* **182:** 1844–1853.