Hpa1 secretion via type III secretion system in *Xanthomonas oryzae* pv. *oryzae*

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**Abstract** In many Gram-negative plant pathogenic bacteria the type III secretion system (TTSS), encoded by *hrp* genes, is essential for pathogenicity in the host and induction of a hypersensitive reaction (HR) in nonhost plants. The expression of *hrp* genes has been suggested to be repressed in complex media, whereas it is induced in planta and under certain in vitro conditions. We recently reported that XOM2 medium allows efficient *hrp* expression by *Xanthomonas oryzae* pv. *oryzae*. In this study, we investigated *hrp*-dependent secretion of proteins by the bacteria in vitro. Using modified XOM2, in which bovine serum albumin was added and the pH was lowered to 6.0, we detected at least 10 secreted proteins and identified one as Hpa1. This is the first evidence of protein secretion via TTSS in *X. oryzae* pv. *oryzae*.

**Key words** *Xanthomonas oryzae* pv. *oryzae* · Type III secretion system · *hrp* · Hpa1

The type III secretion system (TTSS) is known to be essential for virulence in animal pathogenic bacteria such as *Shigella*, *Salmonella* and *Yersinia* spp. (Hueck 1998). The supramolecular structure of TTSS, termed the needle complex (Kubori et al. 2000), causes infection by injecting effectors into host cells. TTSS is also conserved in plant pathogenic bacteria (Alfano and Collmer 1997). It is encoded in an *hrp* gene cluster and is required to elicit a hypersensitive response (HR) in nonhost or resistant host plants and for pathogenicity in susceptible hosts. TTSS is thought to be employed in bacterial attachment, injection of virulence factors such as avirulence gene products and harpins, or both. It has been suggested that the expression of *hrp* genes is repressed in complex media, whereas it is induced in planta and under certain conditions in vitro (Brito et al. 1999; Wengelnik et al. 1996). In some bacteria pathogenicity-related proteins were shown to be secreted via the TTSS to culture using *hrp*-inducing media (Arlat et al. 1994; Bogdanove et al. 1996; Charkowski et al. 1998; He et al. 1993; Kim and Beer 1998; Rossier et al. 1999).

*Xanthomonas oryzae* pv. *oryzae* is a Gram-negative phytopathogenic bacterium that causes blight in rice (Swings et al. 1990). The *hrp* cluster has been sequenced, and some of the *hrp* genes have been shown to have homology with the genes encoding components of the TTSS. It is speculated, therefore, that some effectors are secreted through this system. The expression of *hrp* genes encoding putative components of TTSS is regulated by HrpXo, a member of the AraC family, and is repressed in complex media as with other bacteria (Oku et al. 1995; Tsuge et al. 2002). Because the induction of *hrp* gene expression in vitro had not been established in *X. oryzae* pv. *oryzae*, detection/identification of the secreted proteins has not yet been achieved. We recently devised an effective *hrp*-inducing medium XOM2 (Tsuge et al. 2002) for the bacterium. We report here the detection of proteins of *X. oryzae* pv. *oryzae* secreted in an *hrpXo*-dependent manner cultured in XOM2 medium and identified one of them as Hpa1.

Some avirulence proteins of *Pseudomonas syringae* pathovars are secreted in culture via the TTSS in a pH-sensitive manner (Van Dijk et al. 1999). A pH-dependent secretion of AvrBs3 through TTSS has also been reported in *X. campestris* pv. *vesicatoria*, and a lower pH (5.0) leads to more efficient secretion (Rossier et al. 1999). Therefore, prior to examining the proteins secreted by *X. oryzae* pv. *oryzae* in culture, we investigated the pH dependence of *hrp* expression in the bacteria. Strain T7174R (Ezuka and...
Horino 1974; Watabe et al. 1993) transformed with a plasmid pHMPIPGUS2 harboring an hrcU::gus fusion gene (Tsuge et al. 2002) was preincubated on NBY agar medium (Vidaver 1967), suspended at OD_{600} = 2.0 with sterilized water, and washed twice. Then, 40 µl of this bacterial suspension was inoculated into 1 ml of the modified XOM2 medium. 

The pH of the medium was adjusted to 5.0, 5.5, 6.0, 6.5, or 7.0 (originally adjusted to pH 6.5 with KOH). After incubation for 15 h at 28°C, 100 µl of the culture was added to an equal volume of 2 X assay buffer (0.1 M phosphate buffer pH 7.0, 0.15% Fe(III)-EDTA, and 5 mM MgCl₂) in which the pH was adjusted to 5.0, 5.5, 6.0, 6.5, or 7.0 (originally adjusted to pH 6.5 with KOH). After incubation for 15 h at 28°C, 100 µl of the culture was added to an equal volume of 2 X assay buffer (0.1 M phosphate buffer pH 7.0, 0.15% β-mercaptoethanol, and 0.2% Triton X-100) containing 2 mM p-nitrophenyl β-D-glucuronide as substrate (Jefferson et al. 1987). One unit of β-glucuronidase (GUS) activity was defined as the amount (in nanomoles) of p-nitrophenol released per hour.

As shown in Fig. 1A, the highest level of GUS-specific activity was obtained at pH 6.0, suggesting that this pH is appropriate for induction of hrp expression. The activity markedly decreased at a lower pH (5.0 and 5.5). The bacterial growth decreased as the pH of the medium was lowered (data not shown). It is likely that the weak biological activity at the low pH led to the decrease in hrp expression. The pH of the medium after incubation was almost the same as that at the beginning of inoculation (data not shown).

To detect hrp-dependent secretory proteins in culture supernatant, XOM2 was modified as follows: (1) the pH was changed to 5.5, 6.0, and 6.5; (2) bovine serum albumin (BSA) (50 µg/ml) was added because Rossier et al. indicated that addition of BSA is necessary to detect X. campestris pv. vescatoria AvrBs3 (Rossier et al. 1999); (3) L-[³⁵S]methionine (0.74 MBq/ml, 20 pmol/ml) (Amersham Biosciences, Buckinghamshire, UK) was added, and the amount of nonlabeled methionine was reduced to 1:100 to improve the efficiency of the utilization of labeled methionine by bacteria, which did not cause inhibition of bacterial growth until at least 15 h after inoculation (data not shown). Strain T7174R, prepared as described above, was used to inoculate 1 ml of the modified XOM2 medium. After a 15-h incubation, bacteria were removed by centrifugation at 10 000 g for 5 min and filtration, and 800 µl of the supernatant was precipitated on ice with 10% (v/v) trichloroacetic acid (TCA). After centrifugation at 14 000 g for 30 min at 4°C, protein precipitates were washed with 100% ethanol and resuspended in 100 µl of Laemmli buffer (Laemmli 1970). Protein samples were boiled for 3 min and separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and ³⁵S-containing proteins were detected by fluorography using ENHANCE (NEN Life Science Products, St. George, UT, USA) (Fig. 1B). As a control, we used the hrpXo mutant 74A4HrpXo (Tsuge et al. 2001) because the gene product is a regulator of other hrp genes (hrpB-F), and disruption of the gene may cause deficient hrp-dependent protein secretion.

As shown in Fig. 1B, we detected bacterial proteins in the supernatant of the culture; more proteins were secreted when the pH of the medium was 6.0, which is consistent with the induction of hrcU expression being most active at this pH (Fig. 1A). More than 10 proteins were detected under these conditions, and some were thought to be secreted hrpXo-dependently because the corresponding bands were absent or extremely weak in samples prepared from cultures incubated with the hrpXo mutant. We confirmed that the proteins detected were not derived from bacterial lysis by comparing the band patterns with those from total cell proteins (data not shown). These results suggest that pH is an important factor for the hrp-dependent secretion of proteins in X. oryzae pv. oryzae.

**Fig. 1.** Effect of pH on induction of hrcU expression (A) and hrp-dependent protein secretion (B). A β-Glucuronidase (GUS) activity of Xanthomonas oryzae pv. oryzae T7174R transformed with a plasmid harboring the hrcU::gus fusion gene was measured after a 15-h incubation in modified XOM2 medium. The pH of XOM2 was adjusted to pH 5.5, 6.0, or 6.5 in the presence of bovine serum albumin (BSA) (50 µg/ml). GUS activity per 10⁸ cfu was examined. The reaction solution was maintained at pH 6.85–7.0 for all samples. The results are means of three independent experiments. B X. oryzae pv. oryzae T7174R (W) and hrpXo mutant 74A4HrpXo (X) were grown at 28°C in modified XOM2 (pH 5.5, 6.0, or 6.5) which contained BSA and L-[³⁵S]methionine. Bacteria were removed by centrifugation and filtration; proteins in the supernatant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and ³⁵S-containing proteins were detected by fluorography.