NHEJ, using screens for genes up-regulated in haploids versus diploids (*22, 23*) or using two hybrid-screens with Lif1p (*24*).

hap3 mutants also showed a high average (mean ± SD) percentile (99.3 ± 0.4%) in diploid microarray analyses (Fig. 1). Preliminary results suggest that diploid *hap3* mutants have modest linear-plasmid transformation defects. However, when diploid *hap3* mutants were transformed with Eco RI–digested pSO98 (see the legend to Fig. 2A) and plated onto SC-Leu to select for precisely rejoined transformants, diploid *hap3* mutant transformation efficiency was not distinguishable from that of wild-type cells, whereas *yku80* mutants had a fivefold-reduced transformation efficiency.

We have shown here that pools of thousands of mutants generated by DNA transformation can be analyzed in parallel. This approach is likely to have many important applications in any genetic screen requiring a plasmid. Furthermore, this approach makes feasible genetic screens for quantitative phenotypes such as NHEJ frequency, mutation rate, and gross chromosomal rearrangement rate, which are physically too labor-intensive to be carried out by conventional screening methods. Finally, databases containing quantitative phenotypic information of this type will provide an important resource for mapping genetic-interaction networks.

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3. The UPTAG and DOWNTAG features allow large numbers of deletion strains to be pooled and analyzed in parallel. The pool of mutants was transformed with either circular or Eco RI–linearized pRS416 and plated onto SC-Ura plates. Genomic DNA was isolated from pooled Ura$^+$ transformants and used as a PCR template to amplify the DOWNTAGs or UPTAGs of the strains present. Genomic DNA from the circular pRS416-transformed cells was amplified with Cy5 (red)-labeled universal primer, whereas that of the Eco RI–linearized pRS416-transformed cells was amplified with Cy3 (green)-labeled universal primers. These fluorescently labeled probes were then cohybridized to a DNA microarray bearing each tag in triplicate. An NHEJ-defective deletion strain would be underrepresented in the Eco RI–linearized plasmid-transformed pool and would have a reduced signal in the Cy3 channel (Web fig. 1B).
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11. Construction of the deletion strain pools: To construct the deletion pools, collections of 4647 *MAT***a** haploid mutants (Research Genetics) or 3546 homozygous diploid mutants (gift of M. Johnston) were pinned as individual 5-mm patches onto YPD plates containing G418 (200 μg/ml) and incubated at 30°C for 3 days. The mutants were scraped into ~300 ml of 15% glycerol. The optical densities at 600 nm (OD_{600}) of the deletion pools were adjusted to 15 OD_{600}/ml with 15% glycerol, and pools were frozen in 1- to 2-ml samples.
12. Competent cell preparation: 2 ml of 15 OD_{600}/ml frozen mutant pool were added to ~300 ml of YPD to obtain an initial starting OD of 0.125 OD_{600}/ml. The culture was then shaken at 30°C for ~5 hours to an OD of 0.5 OD_{600}/ml. The culture was harvested, and then washed once in water and once in 0.1 M LiOAc. The cells were then adjusted to 0.2 OD_{600}/μl in about 800 μl of 0.1 M LiOAc. Yeast transformation: For each transformation, 150 μl of competent cells were used. The number of colonies required for reproducible hybridization results was 10^5 to 10^6. Circular pRS416 and Eco RI–digested pRS416 were used to transform each deletion pool (Web table 1). Ten microliters of sheared, heat-denatured herring sperm DNA (10 mg/ml) was added to the transforming DNA, followed by the addition of 150 μl of competent cells and 444 μl of 45.4% polyethylene glycol (PEG-3350) in 0.1 M LiOAc. Cells were rotated in a 30°C incubator for 30 min. Dimethyl sulfoxide (67 μl) was then added and cells were heat-shocked at 42°C for 15 min. Cells were washed and resuspended in 4.2 ml of water. The transformation reaction was then plated onto 10 150-mm SC-Ura plates and incubated at 30°C for 2 to 3 days.
13. Genomic DNA preparation: The cell suspensions from 10 plates (~30 ml) were pooled and then mixed thoroughly by vortexing, and 10 to 15 OD units of cell pellet was used for genomic DNA preparation. The rest of the pellet was resuspended in 15% glycerol and stored at −70°C. Genomic DNA was prepared as described (*25*), followed by ribonuclease A digestion, phenol chloroform extraction, and ethanol precipitation.
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27. We thank all members of the *Saccharomyces* Genome deletion project consortium [see (*1*)] for the collection of deletion mutants. We thank I. Celic and A. IJpma for encouragement and technical assistance; T. Gunatilaka and D. Zack for help with scanning; N. Bachman, C. Connelly, M. Lee, and S. Sookhai-Mahadeo for help with deletion pools; and M. Johnston and E. Winzeler for strains and helpful discussions. E. Bolton, B. Cormack, D. Koshland, J. Pevsner, F. Spencer, and D. Yuan provided helpful discussions and J. Haber, S. Åstrom, M. Frank-Vaillant, and S. Marcand provided discussions and shared unpublished results. Supported by NIH grants HG01627 and GM36481.

23 August 2001; accepted 17 October 2001
Published online 8 November 2001;
10.1126/science.1065672
Include this information when citing this paper.

Role of the Hrp Pilus in Type III Protein Secretion in *Pseudomonas syringae*

Qiaoling Jin and Sheng-Yang He*

Bacterial surface appendages called pili and needle-like filaments are associated with protein and/or DNA transfer to recipient plant, human, or bacterial cells during pathogenesis or conjugation. Although it has long been suspected that pili function as a conduit for protein or DNA transfer, direct evidence has been lacking. The Hrp pilus of *Pseudomonas syringae* is assembled by the type III secretion system. We used an in situ immunogold labeling procedure to visualize the extrusion of an effector protein, AvrPto, from the tip of the Hrp pilus, providing direct evidence that a bacterial pilus can function as a conduit for protein delivery.

Pili are nonflagellate filamentous surface appendages produced by virtually every species of Gram-negative bacterium. Pili are known to function as a means of attaching bacteria to various surfaces, often playing a crucial role in bacterial infection of humans and plants. Several pili are also required for delivery of effector proteins and DNA into recipient host or bacterial cells (*1–4*). Although the long and thin pili appear to be especially suited for long-distance protein and DNA delivery, direct evidence showing the extrusion of effector proteins or DNA from the tip of a pilus has never been shown.

The Hrp pilus of *Pseudomonas syringae* pv. *tomato* strain DC3000 (hereafter *Pst* DC3000) is assembled by the *hrp* gene–encoded type III secretion system (*4–8*). This pilus is composed of a major subunit, the HrpA protein. The *hrpA* gene is required directly or indirectly for the secre-

Department of Energy Plant Research Laboratory and Department of Plant Biology, Michigan State University, East Lansing, MI 48824, USA.

*To whom correspondence should be addressed. E-mail: hes@msu.edu

tion of effector proteins, such as AvrPto and HrpW, in culture (*9*). In situ immunogold labeling studies reveal that during active type III secretion, AvrPto and other effectors are localized specifically along the entire length of the Hrp pilus but not randomly in the extracellular space or along flagella (*10*, *11*). Type III secretion thus occurs only at specific sites where the Hrp pilus is assembled. The deposition of AvrPto and other effectors along the entire length of the Hrp pilus suggests that secretion of effector proteins occurs while the Hrp pilus is being constructed, presumably because the expression of effector genes (such as *avrPto*) and type III secretion/Hrp pilus assembly genes (*hrp*/*hrc*) is co-regulated in *Pst* DC3000, owing to the presence of a common "harp box" regulatory motif in the promoters of these genes (*12*–*14*). This co-regulation made it impossible to determine whether effector proteins exit from the tip or the base of a continuously growing pilus (*11*).

In this study, we uncoupled the expression of the *avrPto* gene from the assembly of the Hrp pilus by placing *avrPto* under the control of the *P. aeruginosa* salicylic acid (SA)–inducible P_G promoter (*15*) in pAVRPTO (*16*). pAVRPTO was introduced into an *avrPto* deletion mutant, *Pst* DC3000Δ*avrPto* (*17*). In *Pst* DC3000Δ*avrPto*(pAVRPTO), AvrPto was produced and secreted in *hrp*-inducing medium only when SA was supplemented (Fig. 1A). As a periplasmic protein control, β-lactamase, expressed constitutively from the *bla* gene of pAVRPTO, was detected independently of SA in the cell fraction but was not released to the medium (Fig. 1B). Thus, in *Pst* DC3000Δ*avrPto*(pAVRPTO), AvrPto production was tightly controlled by SA, and the extracellular release of AvrPto was not due to nonspecific leakage.

We next used in situ immunogold labeling to determine whether AvrPto is still localized specifically to Hrp pili under the SA induction condition (*18*). No significant level of gold particles (indicative of secreted AvrPto) was found when DC3000Δ*avrPto*(pAVRPTO) was grown in *hrp*-inducing medium without SA (Fig. 2A). In contrast, when *Pst* DC3000Δ*avrPto*(pAVRPTO) was grown in the *hrp*-inducing medium supplemented with SA for 4 hours, AvrPto was abundantly secreted and was localized almost exclusively along a subpopulation of Hrp pili (Fig. 2B). We counted 95 Hrp pili that were attached to bacteria and labeled by the AvrPto antibody; they fell into two categories. Eighty-two Hrp pili were labeled along the entire length of the Hrp pilus. This category included those Hrp pili that were labeled uniformly or with more gold particles at the distal end, or occasionally at the proximal end. We also observed 13 Hrp pili with gold particles deposited along the Hrp pilus, except for the base (about one-tenth of the entire pilus length; occasionally close to one-third of the length). Thus, under the SA induction condition, AvrPto was still specifically localized to Hrp pili. However, the finding of the second category of labeling pattern, albeit at a low frequency, suggested that the assembly of at least some Hrp pili had already begun before secretion of AvrPto, even though bacteria were exposed to *hrp*-inducing medium and SA at the same time.

We next examined the localization of AvrPto under conditions in which the Hrp pilus assembly was purposely induced first in the *hrp*-inducing medium for 3 hours, before a "chase" induction of AvrPto expression with SA for 1 hour (*18*). We reasoned that if AvrPto travels through the Hrp pilus and exits from the tip [the conduit model (*11*)], we should observe an increased number of Hrp pili with AvrPto localized near the tip. In contrast, if AvrPto exits from a hypothetical channel near the base of the Hrp pilus [the "guiding filament" model (*11*)], we should observe an increased number of Hrp pili with AvrPto localized near the base. We counted 100 Hrp pili that were attached to bacteria and labeled with the AvrPto antibody from four independent experiments. We found that the AvrPto labeling was drastically shifted to the tip portion of the Hrp pilus, as compared with that when the Hrp pilus assembly and AvrPto synthesis were co-induced (Fig. 2B). Seventy-two Hrp pili were labeled primarily along the top portion, among which were 42 Hrp pili with gold particles along the tip one-third portion of the Hrp pilus (Fig. 2C) and 30 Hrp pili with gold particles mainly along the top two-thirds of the Hrp pilus (Fig. 2D). Only 28 Hrp pili were labeled along the entire length. No Hrp pili with gold particles labeled exclusively at the base were found. Because each Hrp pilus was presumably at

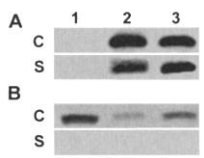

Fig. 1. Immunoblot analysis of AvrPto synthesis and secretion in *Pst* DC3000Δ*avrPto* (pAVRPTO). Bacteria were grown in *hrp*-inducing medium for 4 hours at 20°C without SA (lane 1), with 350 μM SA for 4 hours (lane 2), or with 350 μM SA only for the last 1 hour (lane 3). Cell (C) and supernatant (S) fractions were separated by centrifugation. Proteins were resolved in 12% SDS-PAGE gel and immunoblotted with AvrPto (**A**) and β-lactamase (**B**) antibodies according to the protocol described previously (*25*).

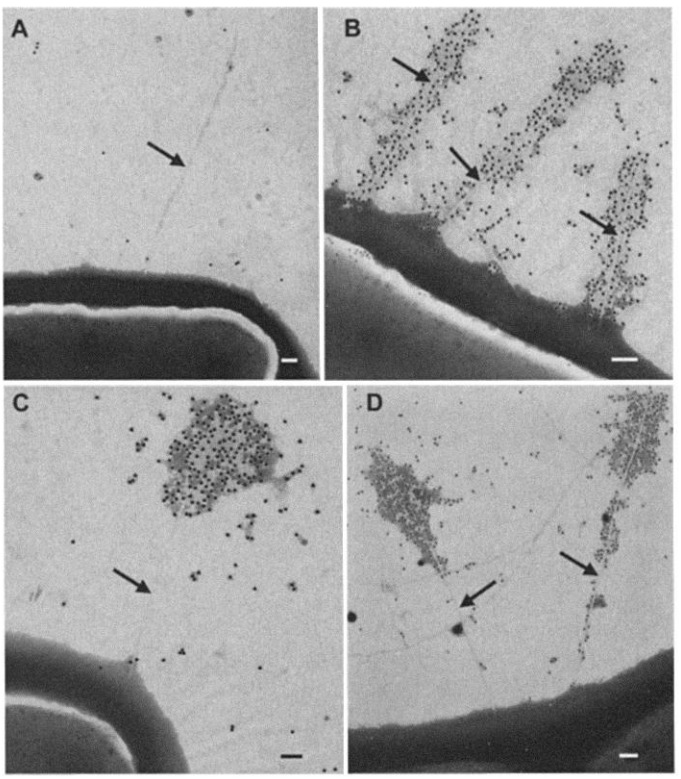

Fig. 2. Representative EM images of *Pst* DC3000Δ*avrPto* (pAVRPTO), immunogold-labeled with the AvrPto antibody. In situ immunogold labeling (*18*) was done after bacteria were grown in *hrp*-inducing medium for 4 hours, supplemented with (**A**) no SA, (**B**) SA for 4 hours, or (**C** and **D**) SA for the last 1 hour (chase induction). The Hrp pilus was not labeled in the absence of SA (A), was labeled throughout the Hrp pilus in the presence of SA for 4 hours (B), or was labeled along the top portion of the Hrp pilus under the chase induction condition (C and D). Dark dots are 15-nm gold particles in (A) and (C) and 10-nm gold particles in (B) and (D). Arrows indicate Hrp pili attached to rod-shaped bacteria (only a portion of the bacterium is shown, surrounded by dark stain). Scale bars, 100 nm.

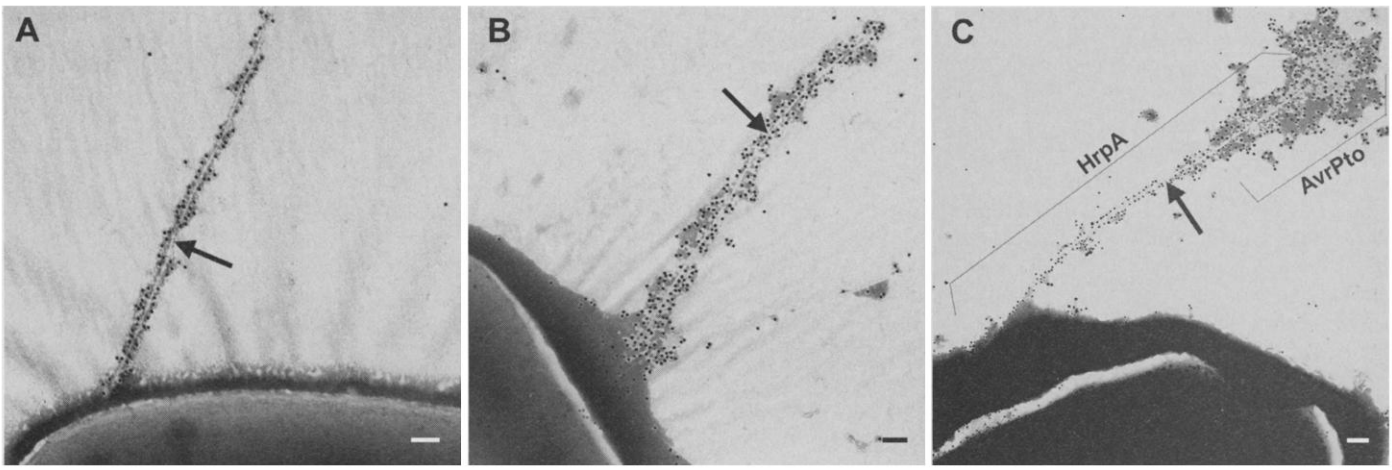

Fig. 3. Representative EM images of *Pst* DC3000Δ*avrPto* (pAVRPTO), immunogold-labeled with the HrpA (**A**) or HrpW (**B**) antibody or dual-labeled with HrpA and AvrPto antibodies (**C**) under the SA chase induction condition (*18*). The HrpA and HrpW antibodies (indicated by 10-nm gold particles) labeled the entire Hrp pili (A and B). In (C), the HrpA antibody (indicated by smaller 10-nm gold particles) labeled the entire Hrp pilus, whereas the AvrPto antibody (indicated by larger 15-nm gold particles) labeled mainly the top portion of the pilus. Arrows indicate Hrp pili. Scale bars, 100 nm.

a distinct assembly stage at the time of SA induction, the uniform localization of AvrPto along the 28 Hrp pili suggested that these Hrp pili were assembled simultaneously with or after SA-induced AvrPto synthesis and secretion. In contrast, the assembly of Hrp pili that were labeled near the top portion must have begun before AvrPto synthesis and secretion. Consequently, the portion of the Hrp pilus assembled before AvrPto secretion was not labeled.

Under the chase immunogold labeling condition, the HrpA and HrpW antibodies still labeled the entire length of the Hrp pili (Fig. 3, A and B). This was best shown when HrpA and AvrPto antibodies were used in dual labeling experiments. Although the HrpA antibody bound to the entire length of the Hrp pilus, the AvrPto antibody labeled mainly along the tip portion of Hrp pili (Fig. 3C). This result is consistent with the fact that in *Pst* DC3000Δ*avrPto*(pAVRPTO), the expression of *hrpA* and *hrpW* genes was under the control of their native harp box–containing promoters, whereas the expression of AvrPto was controlled by SA.

The drastic shift of AvrPto localization toward the tip of the Hrp pilus under the chase immunogold labeling condition directly supports the conduit model (*11*). The demonstrated role of the Hrp pilus as a conduit for protein secretion has important ramifications for further study of the type III secretion mechanism, because any future model must take into account the fact that effector proteins travel inside the Hrp pilus. The conduit function of the Hrp pilus also provides support for the postulated shared mechanism of type III secretion and flagellar assembly, which involves the export of partially folded flagellin through an internal channel of a growing flagellum filament before assembly at the tip (*19*, *20*).

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16. A fragment of ~3 kb containing the P_G promoter was cut out from pKMY319 (*15*) and inserted into the Eco RI and Pst I sites of pUCP19 (*21*), resulting in plasmid pUCP19SA. The *avrPto* gene without the harp box promoter sequence was amplified by polymerase chain reaction from *Pst* DC3000 with the following primers (restriction sites are underlined): 5′-CTGATCTAGA(Xba I)AGTGGCGTCATAAATGCTG-3′ and 5′-ACTTCTCGAG(Xho I)TCATTGCCAGTTACGGTACG-3′, and cloned into pUCP19SA, resulting in plasmid pAVRPTO.
17. An 858–base pair (bp) DNA fragment upstream of the *avrPto* start codon was amplified from *Pst* DC3000 with the following oligonucleotides: 5′-GACTCTAGA(Xba I)TGTGCTTTGCCTATGGCCTGC-3′ and 5′-GCACTGCAG(Pst I)GTATACCCTCTTTAGTACAG-3′. A 798-bp DNA fragment downstream of the *avrPto* stop codon was amplified with the following primers: 5′-GACTGAGCTC(Sac I)TGTCAAGCCGTGTGTGGCG-3′ and 5′-GCGAATTC(Eco RI)AGAGGTTGATGCATGGGTCG. The 815-bp open reading frame (ORF) of the *aph* gene was amplified from mini-Tn*5xylE* (*22*) with the following primers: 5′-AAGCTGCAG(Pst I)ATGAGCCATATTCAACGGG-3′ and 5′-GACTGAGCTC(Sac I)TTAGAAAAACTCATCGAGC-3′. These three fragments were ligated together and inserted into the Xba I and Eco RI sites of pRK415 (*23*). The resulting plasmid was introduced into *Pst* DC3000 by electroporation. An *avrPto*-deficient strain, *Pst* DC3000Δ*avrPto*, was obtained by marker-exchange mutagenesis according to a standard protocol (*24*).
18. In situ immunogold labeling followed a protocol described previously (*10*, *11*), except for the following modifications. After *Pst* DC3000Δ*avrPto*(pAVRPTO) was resuspended in *hrp*-inducing medium, a 9-μl droplet was placed on each electron microscopy (EM) grid. For samples without SA addition, 1 μl of *hrp*-inducing medium was added to each droplet. For samples with SA, 1 μl of 3.5 mM SA dissolved in *hrp*-inducing medium was added at 0 hours for a 4-hour induction or at 3 hours for a 1-hour chase induction. Bacteria were grown at 20°C for a total of 4 hours. For single immunogold labeling experiments, a rabbit antibody to AvrPto was used as the primary antibody, and a goat anti-rabbit immunoglobulin G (IgG) gold conjugate (either 10-nm or 15-nm gold conjugate) was used as the secondary antibody. In dual immunogold labeling experiments, a mouse antibody to HrpA and a rabbit antibody to AvrPto were used as primary antibodies. The secondary antibodies were anti-mouse IgG 10-nm gold conjugate and anti-rabbit IgG 15-nm gold conjugate.
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26. We thank J. Walton and members of our laboratory for critical reading of the manuscript; A. Collmer for antibodies; I. Brown, J. Mansfield, and R. Koebnik for useful discussions and sharing of manuscripts; and K. Bird for assistance in preparation of this paper. Supported by grants from the U.S. Department of Energy and U.S. Department of Agriculture.

19 September 2001; accepted 29 October 2001