An Amino Acid Substitution at Position 740 in $\sigma^{70}$ of Ralstonia solanacearum Strain OE1-1 Affects Its In Planta Growth

Ayami Kanda,1† Kazuhiro Tsuneishi,1 Ai Mori,1 Kouhei Ohnishi,2 Akinori Kiba,1 and Yasufumi Hikichi1*

Laboratory of Plant Pathology & Biotecnology, Kochi University, Nankoku, Kochi 783-8502, Japan, and Research Institute of Molecular Genetics, Kochi University, Nankoku, Kochi 783-8502, Japan2

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Ralstonia solanacearum strain OE1-1 shows virulence on tobacco, as well as tomato and eggplant plants (17). OE1-1 possesses hrp genes, similar to R. solanacearum strain GM11000, which is virulent on tomato plants and elicits a hypersensitivity response in infiltrated tobacco leaves (2, 5, 19, 34). After vigorous proliferation in intercellular spaces, OE1-1 invaded xylem vessels and then systemically infected the whole plant (17, 32, 33). The hrp mutants of OE1-1 lose their ability not only to proliferate in intercellular spaces but also to systemically infect the whole plant. Therefore, it is thought that proliferation of the bacteria in intercellular spaces after invasion of roots qualitatively control bacterial virulence.

The hrp genes encode proteins that construct the type III secretion system. In R. solanacearum, expression of hrp genes is regulated by the HrpB protein (2, 13, 34). Screening of genes controlled by HrpB has isolated many candidates for type III effector genes in R. solanacearum (9, 25). Moreover, by in vivo expression technology, genes expressed in R. solanacearum K60-infected tomato plants were isolated (6). However, the involvement of these candidates in the bacterium-host plant interactions remains to be elucidated. Furthermore, analysis by in vivo expression technology indicates that genes other than hrp and type III secretion system effector genes are involved in bacterial virulence (6, 7).

To elucidate the mechanism of vigorous growth of OE1-1 in roots in this study, we first selected mutants that lack systemic infectivity and do not provoke disease in tobacco plants. The suicide vector pUTSm/Sp (10) containing mini-Tn5, which includes the spectinomycin resistance gene, was transferred from Escherichia coli HB101 (Takara, Ohtsu, Japan) to OE1-1 by conjugation with E. coli HB101(pRK2013) (12). The roots of 8-week-old tobacco plants (Nicotiana tabacum cv. Bright Yellow) were soaked in a bacterial suspension ($1.0 \times 10^8$ CFU/ml) of 421 spectinomycin-resistant mutants derived from OE1-1 for 30 min, and then inoculated plants were grown in water culture pots (Yamato water culture pot no. 1; Yamato Plastic Co. Ltd., Tokyo, Japan) with one-fifth-strength Hoagland’s solution in a growth room at 25°C under 10,000 lx for 16 h/day (root dipping) (17). Each assay was repeated in five successive trials, and within each trial we treated five plants with each strain. Only one mutant, OE1101, lacked the ability to cause wilt in tobacco plants. Seven days after inoculation by root dipping, the stems of five tobacco plants were cut into three pieces with razor blades (Fig. 1). The cut site of each piece was pressed onto Hara-Ono medium (15) for OE1-1 and onto Hara-Ono medium containing spectinomycin at 50 $\mu$g/ml for OE1101, and the media were incubated at 30°C for 3 days (plate-printing assay) (20). Though OE1-1 was detected at some sites, the mutant was never detected at sites (sites A and B) beyond the bacterium-inoculated area (site C). These results indicate that the mutant cannot systemically infect tobacco plants, leading to the loss of its ability to cause wilt in tobacco plants.

Southern blot analysis with Sm$/^\prime$/Sp$^\prime$ derived from pUTSm/Sp as the probe showed that mini-Tn5 was inserted at one site in the genomic DNA of OE1101 (data not shown). Nucleotide sequence analysis of pMINE, in which 3.4 kbp of genomic DNA of OE1101 including $\text{Sm}^\prime$/Sp$^\prime$ was ligated into pUC118, showed that the site was inserted into the pcoD (which encodes $\sigma^{70}$) region of the mini-Tn5 Sm$/^\prime$/Sp$^\prime$ at nucleotide position 2217 from the start codon (GTG). The bacterial RNA polymerase holoenzyme consists of a catalytic core enzyme with a sigma factor conferring on the holoenzyme the ability to initiate promoter-specific transcription (14). The primary sigma factor in E. coli, $\sigma^{70}$, recognizes promoters that are needed for the transcription of general housekeeping genes and is responsible for most of the transcription during exponential growth (29). $\sigma^{70}$ of OE1101 was predicted to be composed of 758 amino acid residues. The amino acid sequences of 19 residues at the C terminus were derived from mini-Tn5 (Fig. 2). $\sigma^{70}$ shares four regions of similarity with primary sigma proteins of other prokaryotes (14, 23). When present in the RNA polymerase holoenzyme, $\sigma^{70}$ sets the start site for transcription by recognizing various DNA elements, and residues within each of the four regions have been shown to interact with sequences

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1 Corresponding author. Mailing address: Faculty of Agriculture, Kochi University, 200 Monobe, Nankoku, Kochi 783-8502, Japan. Phone: 81-88-864-5218; Fax: 81-88-864-5200. E-mail: yhikichi@cc.kochi-u.ac.jp.

† Present address. National Agricultural Research Center, Tsukuba, Ibaraki 305-8666, Japan.

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in promoter DNA (4, 8, 11, 16, 26). The majority of E. coli promoters have −10 and −35 DNA elements, which are contacted by σ70 regions 2 and 4, respectively (8, 26). Specific base recognition of the −35 sequences arises through a DNA-binding helix-turn-helix (HTH) motif in region 4.2 (21, 27, 28).

The Arg740 was located adjacent to the C terminus of region 4.2 and the DNA binding HTH motif of OE1-1 σ70.

To analyze the effect of the Arg740 substitution in σ70 on bacteria virulence, 3.0-kbp and 361-bp DNA fragments were PCR amplified from genomic DNA of OE1-1 with primers Xba-rpoD (5′-GCTCTAGACCTCGGTGAGCAGCTGCAGCAGC-3′) with an added XbaI site (underlined) and Mutant-FW (5′-TCCGAACCGCCGTTACACAGATGTTGCGC-3′) and primers Mutant-RV (5′-AGGCGCTGCGCAGGCGCGAGGC-3′) with HindIII site (underlined), respectively. Each PCR product was mixed and PCR amplified with the Xba-rpoD and Hind-rpoD primer pairs. The resultant HindIII-digested 3.3-kbp DNA fragment was ligated into XbaI- and HindIII-digested pK18mobsacB (30) to create pK18mobsacBRS3. This plasmid was electroporated into OE1-1 cells (1, 33), and kanamycin-resistant and sucrose-sensitive recombinants were selected. The recombinant was incubated in a rich medium (PS medium) (17) for 6 h and a kanamycin-sensitive and sucrose-resistant recombinant, OE102, was selected. DNA sequencing of PCR-amplified DNA fragments with Xba-rpoD and Hind-rpoD as primers was performed to verify the only Arg740Cys substitution in σ70 (data not shown). OE1102 grew in both PS medium and a minimal medium (Boucher medium) (5), similar to OE1-1, indicating that the Arg740Cys change in σ70 did not affect in vitro bacterial growth (data not shown).

Reduced sap flow caused by the presence of a large number of bacterial cells and exopolysaccharide (EPS) slime produced by the bacteria in some xylem vessels lead to extensive wilting in plants (31). EPS I content was quantified by measuring hexosamine with the Elson-Morgan reaction (18). The EPS I productivity of OE1102 was 448 μg of polymeric (>14-kDa) hexosamine/mg cell protein, similar to that of the wild type (432 μg/mg).

OE1-1 infiltration of tobacco leaves induced necrotic lesions, which is dependent on hrrp genes, at the sites of infection 60 h after inoculation (19). OE1102 induced necrotic lesions in the infiltrated area of tobacco leaves at 60 h after inoculation, as well as the parent strain.

The hrrpY and popA genes encode a protein constructing hrp pili and the type III effector protein PopA, respectively. To analyze the influence of an Arg740Cys substitution in σ70 on the expression of rpoD, hrrpY, and popA by reverse transcription-PCR, total RNA was isolated from five of each set of tobacco leaves at 0, 1, 3, and 6 h after infiltration with 50 μl bacterial suspension (1.0 × 10^8 CFU/ml) of R. solanacearum strains, and DNase I (Applied Biosystems, Tokyo, Japan) treatment was used to remove the genomic DNA from the RNA preparation (20). rpoD, hrrpY, and popA cDNA fragments were synthesized from total RNA (6 μg) with primers rpoD-FW (5′-CGCCCCTTCGTTACATGCTG-3′), hrrpY-Bam (5′-GCGGGATCTCTTTGAGTTGGTT-3′) and popA-SQ (5′-GTTGGCACCGCTGACAGG-3′), respectively. PCR was performed with primers rpoD-FW and rpoD-RV (5′-GGAATTCATATGGCAGGCGTTC-3′) for the amplification of a 370-bp DNA fragment specific to rpoD, hrrpY-Bam and Nde-hrrpY (5′-GGGAATTCATATGGCAGGCGTTC-3′) for the amplification of a 370-bp DNA fragment specific to hrrpY, and PopA-SQ (5′-GTTGGCACCGCTGACAGG-3′) and popA-SQ+ (5′-GGAATTCATATGGCAGGCGTTC-3′) for the amplification of a 320-bp DNA fragment specific to popA. When the RNA treated with DNase I was used as the template in PCRs, no product was observed. The expression of mutated rpoD in OE1102-infiltrated tobacco leaves was constitutively detected, as well as that of rpoD in OE1-1-infiltrated leaves (Fig. 3). Furthermore, expression of hrrpY and popA in OE1102-infiltrated tobacco leaves was detected immediately after inoculation and 3 h after infiltration, respectively, similar to that in OE1-1-infiltrated leaves (Fig. 3). These results indicated that OE1102 retained its activity to express hrrp and popA genes, similar to OE1-1.

Tobacco plants inoculated with OE1-1 wilted 7 days after inoculation. In contrast, OE1102 lacked virulence on tobacco plants, as did OE1101. To analyze the virulence of R. solanacearum strains on other solanaceous plants, 8-week-old tomato (Lycopersicon esculentum cv. Ohgata-Fukuju) and egg-
plant (Solanum melongena cv. Senryo-nigou) plants were inoculated with a bacterial suspension at $1.0 \times 10^6$ CFU/ml by root dipping. Though OE1-1 showed virulence on eggplant and tomato plants 7 days after inoculation, OE1101 and OE1102 lacked virulence on both solanaceous plants. These results indicate that an Arg740Cys substitution in $\sigma^{70}$ leads to a loss of bacterial virulence.

To analyze the bacterial population in roots, roots were excised daily from five of each set of tobacco plants, from 0 to 5 days after inoculation with bacteria, and ground with a mortar and pestle. The original solution and 10-fold serial dilutions of it were spread onto three plates of Hara-Ono medium. The colonies were counted after 2 days of incubation at 30°C. The population of OE1-1 in roots increased to $7.0 \times 10^7$ CFU/g 5 days after inoculation (Fig. 4). In contrast, the population of OE1102, in roots drastically decreased to $3.0 \times 10^5$ CFU/g 5 days after inoculation. The plate-printing assay showed that OE1102 was detected in the only bacterium-inoculated area, as well as OE1101. These findings indicate that an Arg740Cys substitution in $\sigma^{70}$ results in loss of the bacterial ability to grow in roots, leading to loss of systemic infectivity.

To test if the rpoD mutation leading to an Arg740Cys substitution is dominant or recessive, the dominant negative phenotype was analyzed with the rpoD mutation located on the plasmid or on the recipient chromosome. DNA fragments of 3.4 kbp were PCR amplified with OE1102 genomic DNA and OE1-1 genomic DNA as templates and XbaI- and HindIII-digested OE1102 genomic DNA and OE1-1 genomic DNA as primers, and the resultant XbaI- and HindIII-digested fragments were ligated into the XbaI and HindIII sites of pUCD3101 (17) to create pUCD3101rpoDRS3 and pUCD3101rpoD, respectively. OE1-1 and OE1102 were transformed with pUCD3101rpoDRS3 and pUCD3101rpoD by electroporation to create kanamycin-resistant transformants OE1103 and OE1104, respectively. Both OE1103 and OE1104 lacked virulence on tobacco plants, similar to OE1102. Furthermore, the plate-printing assay with Hara-Ono medium containing kanamycin at 50 $\mu$g/ml showed that both transformants were never detected at sites beyond the bacterium-inoculated area, suggesting that both OE1103 and OE1104 lacked systemic infectivity. Transformants of OE1-1 and OE1102 with pUCD3101 showed the same virulence phenotype as OE1-1 and OE1102, respectively. These results indicate the negative dominance of mutant $\sigma^{70}$ over the wild-type protein.

The presence of a good match to both the $-10$ and $-35$ canonical sequences of promoter DNA is usually sufficient for E. coli $\sigma^{70}$ to recognize a promoter without the aid of additional factors. However, activation factors can be needed when either sequence element deviates significantly from the consensus. Many activators in E. coli work through class I or class II activation mechanisms (3, 22). Class I activators interact with residues in the C-terminal domain of the $\alpha$ subunit of polymerase. Class II activators interact with residues in $\sigma^{70}$ region 4, on the basis of mutations in $\sigma^{70}$ that selectively eliminated the function of an activator without altering basal transcription. These activator-specific mutations cluster in two regions of $\sigma^{70}$: a segment at the beginning of the first helix (amino acids 573 to 580) and a segment downstream from the second helix (amino acids 584 to 598) of the HTH motif in E. coli $\sigma^{70}$ region 4.2. The five basic residues, including Arg599, in a narrow region of E. coli $\sigma^{70}$ (residues 590 to 603) are implicated in activation, and the residues are either in the C terminus of a long recognition helix that includes residues recognizing the $-35$ hexamer region of the promoter or in the subsequent loop (24). The Arg599 residue in $\sigma^{70}$ of E. coli, which corresponds to Arg740 in $\sigma^{70}$ of OE1-1, is reportedly the contact point with the cyclic AMP receptor protein, which regulates catabolic genes under aerobic conditions. Therefore, an Arg740Cys substitution may lead to changes in interactions between the class II activators and $\sigma^{70}$, resulting in differences in the expression of genes that are required for vigorous in planta growth of the bacteria. The effects of interactions between the class II activators and $\sigma^{70}$ on the expression of genes involved in bacterial in planta growth remains to be elucidated.

Taken together, our results indicate that the potential of $\sigma^{70}$ with an Arg740Cys substitution to suppress bacterial growth in planta may lead to elucidation of the mechanism of bacterial growth in plants, which plays a key role in bacterial virulence.

**Nucleotide sequence accession numbers.** rpoD nucleotide sequences of R. solanacearum strains OE1-1 and OE1101, which was derived from OE1-1 with synthetic transposon mini-Tn5 Sm/Sp, were assigned to the DDJ database under accession numbers AB358977 and AB445124, respectively.

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