

Flagellin Glycosylation Island in *Pseudomonas syringae* pv. *glycinea* and Its Role in Host Specificity

Kasumi Takeuchi,^{1,2} Fumiko Taguchi,¹ Yoshishige Inagaki,¹ Kazuhiro Toyoda,¹
Tomonori Shiraiishi,¹ and Yuki Ichinose^{1*}

Laboratory of Plant Pathology & Genetic Engineering, Faculty of Agriculture, Okayama University, Tsushima-naka 1-1-1, Okayama 700-8530,¹ and Laboratory of Microorganism Genetic Resources, Genebank, National Institute of Agrobiological Sciences, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602,² Japan

Received 30 May 2003/Accepted 17 August 2003

The deduced amino acid sequences of the flagellins of *Pseudomonas syringae* pv. *tabaci* and *P. syringae* pv. *glycinea* are identical; however, their abilities to induce a hypersensitive reaction are clearly different. The reason for the difference seems to depend on the posttranslational modification of the flagellins. To investigate the role of this posttranslational modification in the interactions between plants and bacterial pathogens, we isolated genes that are potentially involved in the posttranslational modification of flagellin in *P. syringae* pv. *glycinea* (glycosylation island); then defective mutants with mutations in these genes were generated. There are three open reading frames in the glycosylation island, designated *orf1*, *orf2*, and *orf3*. *orf1* and *orf2* encode putative glycosyltransferases, and mutants with defects in these open reading frames, Δ *orf1* and Δ *orf2*, secreted nonglycosylated and slightly glycosylated flagellins, respectively. Inoculation tests performed with these mutants and original nonhost tobacco leaves revealed that Δ *orf1* and Δ *orf2* could grow on tobacco leaves and caused symptom-like changes. In contrast, these mutants failed to cause symptoms on original host soybean leaves. These data indicate that putative glycosyltransferases encoded in the flagellin glycosylation island are strongly involved in recognition by plants and could be the specific determinants of compatibility between phytopathogenic bacteria and plant species.

Flagellin is an essential component of the flagellum filament of bacteria. In recent years, there has been increasing evidence that flagellin is posttranslationally glycosylated (4, 20). However, workers have just started to elucidate the molecular basis of this modification. Thus, little is known about its biological significance.

The isolates of the phytopathogenic bacterium *Pseudomonas syringae* can be classified into more than 50 pathovars on the basis of virulence for host plant species. In a previous study, *P. syringae* flagellin was found to elicit hypersensitive reactions (HR) in nonhost plants (27). Several studies to characterize elicitor flagellins have been performed with *Pseudomonas avenae* (*Acidovorax avenae*) (5) and *P. syringae* pv. *tabaci* (7). These flagellins have been reported to induce plant defense responses in cultured cells of rice (5), tomato, and *Arabidopsis thaliana* (7). A 22-amino-acid peptide (flg22) of a conserved domain near the N terminus based on the sequence of *Pseudomonas aeruginosa* flagellin has been reported to induce plant defense responses but not hypersensitive cell death (7). Thus, flg22 is known as a general elicitor of an innate immune response in plants (10). However, flagellins from *P. avenae* (5) and flagellins from *P. syringae* pv. *glycinea* and *P. syringae* pv. *tomato* (27) are known to induce plant cell death in nonhost rice and tobacco plants, respectively. These results indicate that the flagellin proteins have roles both as nonspecific elicitors of general defense responses and as specific elicitors of

HR cell death in defined plant-bacterium interactions. Furthermore, a flagellin-defective mutant (Δ *fliC*) of *P. syringae* pv. *tabaci* lost both the ability to cause HR in nonhost tomato plants (24) and virulence for host tobacco plants (13). These results suggest that flagella are indispensable for establishing both incompatible and completely compatible relationships.

It has recently been reported that the deduced amino acid sequences of the flagellin proteins of *P. syringae* pv. *tabaci* and *P. syringae* pv. *glycinea* are identical; however, the flagellin protein of *P. syringae* pv. *glycinea* caused cell death in nonhost tobacco cells, whereas that of *P. syringae* pv. *tabaci* did not (27). Actually, the flagellin proteins of these two pathovars were shown to be glycosylated (28). Furthermore, a study performed with a Δ *fliC* mutant of *P. syringae* pv. *tabaci* and mutants with complements of *fliC* genes of different pathovars showed that any flagellin modified in a *P. syringae* pv. *tabaci*-specific manner did not induce HR death of tobacco cells (28). These results suggest that posttranslational modification of the flagellin determines the ability to induce HR. Recently, a genomic island that is necessary for flagellin glycosylation in *P. aeruginosa* was identified directly upstream of the *fliC* gene (1). This report provided a clue to the identity of the gene(s) involved in the posttranslational modification of the flagellin protein of *P. syringae*. It has also been found that part of a sequence homologous to the glycosylation island of *P. aeruginosa* is present upstream of *fliC* in *P. syringae* pv. *tabaci* (24).

Here, we describe genetic and functional analyses of the genes in the glycosylation island involved in the posttranslational modification of flagellin in *P. syringae* pv. *glycinea*. In particular, we found that the putative glycosyltransferases determined the ability to induce HR in nonhost tobacco plants

* Corresponding author. Mailing address: Laboratory of Plant Pathology & Genetic Engineering, Faculty of Agriculture, Okayama University, Tsushima-naka 1-1-1, Okayama 700-8530, Japan. Phone and fax: (81) 86-251-8308. E-mail: yuki@cc.okayama-u.ac.jp.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics ^a	Reference or source
<i>E. coli</i> strains		
DH5a	<i>F</i> ⁻ λ ⁻ ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) <i>U169 recA1 endA1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 thi-1 gyrA relA1</i>	Takara, Kyoto, Japan
S17-1	<i>thi pro hsdR hsdM⁺ recA [chr::RP4-2-Tc::Mu-Km::Tn7]</i>	22
<i>P. syringae</i> pv. <i>glycinea</i> strains		
race 4	Wild type, Nal ^r	A. Collmer
race 4-d1	race 4 Δ <i>orf1</i>	This study
race 4-d2	race 4 Δ <i>orf2</i>	This study
race 4-d3	race 4 Δ <i>orf3</i>	This study
Plasmids		
pUC18	2.7-kb cloning vector, Ap ^r	Takara, Kyoto, Japan
pUCSac1	1.8-kb <i>SacI-SacI</i> fragment in pUC18, Ap ^r	This study
pUCSac2	13.4-kb <i>SacI-SacI</i> fragment in pUC18, Ap ^r	This study
pUCSal1	1.5-kb <i>SalI-SalI</i> fragment in pUC18, Ap ^r	This study
pCR-blunt II-TOPO	3.5-kb cloning vector for PCR blunt end product, Km ^r	Invitrogen
pK18 <i>mobsacB</i>	Small mobilizable vector, Km ^r , sucrose sensitive (<i>sacB</i>)	22
pM1	2.18-kb chimeric PCR product deleting <i>orf1</i> cloned into pK18 <i>mobsacB</i> at <i>EcoRI</i> site, Km ^r	This study
pM2	1.79-kb chimeric PCR product deleting <i>orf2</i> cloned into pK18 <i>mobsacB</i> at <i>EcoRI</i> site, Km ^r	This study
pM3	1.69-kb chimeric PCR product deleting <i>orf3</i> cloned into pK18 <i>mobsacB</i> at <i>EcoRI</i> site, Km ^r	This study

^a Ap^r, ampicillin resistance; Km^r, kanamycin resistance, Nal^r, nalidixic acid resistance.

and also affected the virulence for host soybean plants. To our knowledge, this is the first report that shows that glycosyltransferases of bacterial flagellin influence pathogenicity for eukaryotes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *P. syringae* pv. *glycinea* (race 4) was grown principally on King's B (KB) medium (15) at 27°C. For the inoculation test, bacteria were cultured in Luria-Bertani (LB) medium with 10 mM MgCl₂ at 25°C. For purification of flagellin proteins, bacteria that had been cultured overnight were incubated in minimal medium containing 10 mM mannitol and fructose as carbon sources for 24 h at 23°C (12). *Escherichia coli* strains were grown on LB medium at 37°C.

The following antibiotics were added to the growth media when appropriate: ampicillin (50 µg/ml), kanamycin (25 µg/ml), and nalidixic acid (30 µg/ml).

Plant material and inoculation procedures. Tobacco (*Nicotiana tabacum* cv. Xanthi NC) and soybean (*Glycine max* cv. Shirofumi) plants were grown in a growth chamber at 25°C and 30 to 40% relative humidity with a 12-h photoperiod. To carry out plant inoculation, *P. syringae* pv. *glycinea* was suspended in 10 mM MgSO₄-0.02% Silwet L77 (OSI Specialties Inc., Danbury, Conn.) at a density of 2 × 10⁸ CFU ml⁻¹. Five-week-old tobacco plants or three-week-old soybean plants were spray inoculated with an airbrush until both the abaxial and adaxial leaf surfaces were uniformly wet. For the investigation of bacterial growth on tobacco leaves, 10 µl of bacteria (2 × 10⁵ CFU) was put on needle-pricked leaves, and the bacterial populations were measured. At different times, leaf disks (diameter, 0.5 cm) were punched out from inoculated areas and homogenized in 10 mM MgSO₄; then serial dilutions of the homogenate were plated on KB agar plates. For the investigation of bacterial growth on soybean leaves, bacteria (2 × 10⁸ CFU ml⁻¹) were spray inoculated as described above. Before the bacterial populations were measured, the surfaces of inoculated soybean leaves were sterilized with 15% hydrogen peroxide.

Isolation of glycosylation island. A genomic DNA library of *P. syringae* pv. *glycinea* was constructed by using a lambda FIX II vector kit (Stratagene, La Jolla, Calif.). Because the glycosylation island in *P. aeruginosa* has been reported to be linked to a flagellin gene (*fljC*) (1), a clone containing the *fljC* gene was isolated by using the *fljC* gene as a probe.

Generation of mutant strains. *P. syringae* pv. *glycinea* strains with each open reading frame (ORF) in the glycosylation island deleted were generated based on homologous recombination by using the method described previously (24). Approximately 1-kb DNA fragments located on each side of the *orf1*, *orf2*, and *orf3* genes were amplified by PCR with primers PIUF (5'-TACGACTCACTA

TAGGGCGT-3') and PIUR (5'-CGGGATCCGGAAGTTACCGTCAAGTA A-3') for the upstream region of *orf1*; primers PIDF (5'-CGGGATCCGTTT AATGCGCTCCAGT-3') and PIDR (5'-GGAGTAGTGTGCATAGGCTT-3') for the downstream region of *orf1*; primers P2UF (5'-TCAGCTCAATGAAGT GCAGG-3') and P2UR (5'-CGGGATCCCTGGAGCGCATTAAACAGGT-3') for the upstream region of *orf2*; primers P2DF (5'-CGGGATCCCTCCGCAA TAATCGCAA-3') and P2DR (5'-CTGCATCGCAAACACATG-3') for the downstream region of *orf2*; primers P3UF (5'-CCGAATTATCGACGTGG CTT-3') and P3UR (5'-CGGGATCCGATGAGTAAACAGGTCAT-3') for the upstream region of *orf3*; and primers P3DF (5'-CGGGATCCGTCATGTTGG TTTGGTTTC-3') and P3DR (5'-TATCTTGTACGCGACCCAGT-3') for the downstream region of *orf3* (underlining indicates artificial *Bam*HI sites) (Fig. 1B). Each amplified DNA fragment for upstream and downstream regions was ligated at the *Bam*HI site, and the resulting fragments were amplified by PCR by using the pair of primers PIUF and PIDR for deletion of *orf1*, P2UF and P2DR for deletion of *orf2*, and P3UF and P3DR for deletion of *orf3*. The PCR products were inserted into pCR-blunt II-TOPO vectors (Invitrogen, Carlsbad, Calif.), and we confirmed the expected deletion of each ORF and the absence of nucleotide substitutions in the amplified regions. The constructs were digested with *Eco*RI for generation of the *orf1* and *orf2* mutants or with *Hind*III for generation of the *orf3* mutant and then introduced into the mobilizable cloning vector pK18*mobsacB* (5.7 kb, Km^r) (22). The resulting plasmids, designated pM1, pM2, and pM3, were electrotransformed into *E. coli* S17-1 and then transferred by conjugation from *E. coli* S17-1 to *P. syringae* pv. *glycinea*. After conjugation, the plasmids were excised on a KB agar plate containing 10% sucrose, and specific deletions were confirmed by PCR by using the pairs of primers described above. The resulting bacteria were designated race 4-d1, race 4-d2, and race 4-d3 for the Δ *orf1*, Δ *orf2*, and Δ *orf3* mutants, respectively.

Motility testing. The motility of each mutant was compared with that of the wild-type strain on semisolid (0.3%) MMMF agar plates as previously described (24).

Growth comparisons. The optical density at 600 nm of each bacterial culture was adjusted to 0.1, and the cultures were grown in LB medium with 10 mM MgCl₂ with shaking at 25°C. The optical density at 600 nm was determined after 3, 6, 9, and 24 h.

Purification of flagellin proteins. Flagellin proteins were purified as previously described (27), with a minor modification. Bacteria that had been cultured overnight were collected by centrifugation at 5,000 × *g* for 10 min. The bacterial pellet was resuspended in phosphate buffer (50 mM sodium phosphate, pH 7.0), and flagella were sheared off from the cells by vortexing for 1 min. Cells and cell debris were removed by centrifugation at 10,000 × *g* for 30 min. The resulting supernatant was filtered through a 0.45-µm-pore-size filter and centrifuged at

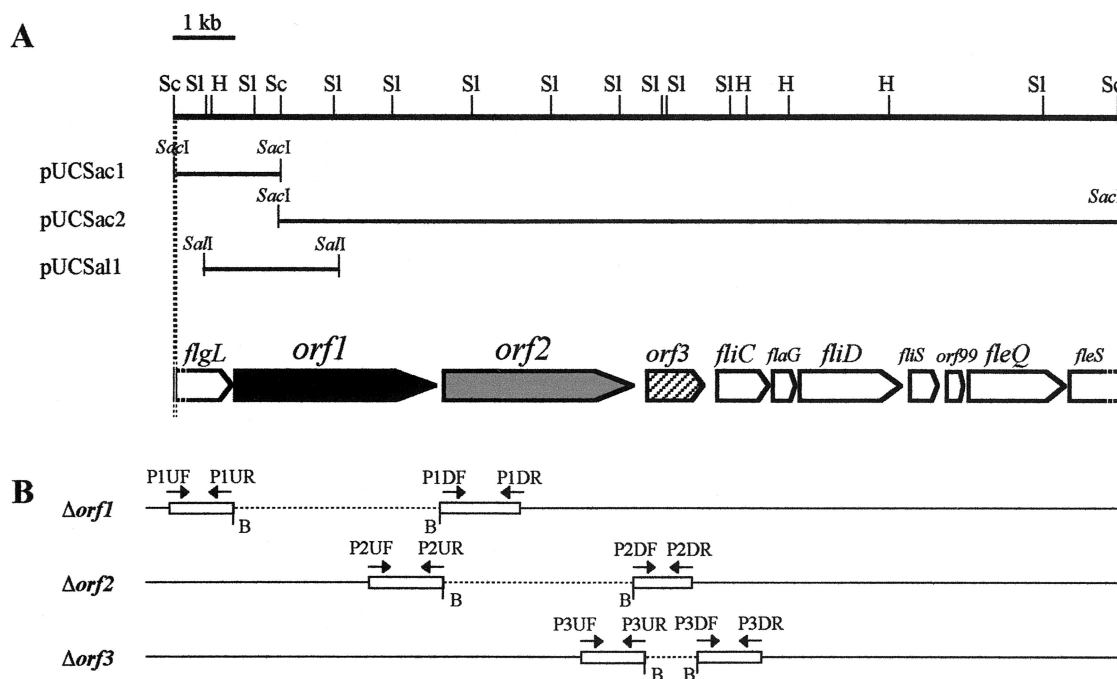


FIG. 1. Flagellum gene structure of *P. syringae* pv. *glycinea* (A) and generation of the $\Delta orf1$, $\Delta orf2$, and $\Delta orf3$ mutants (B). Restriction sites in the flagellum gene cluster and fragments subcloned into the plasmid vector are indicated by horizontal bars. Pentagons labeled with gene designations represent ORFs found in the genomic clone. The glycosylation islands composed of three ORFs are indicated by black (*orf1*), gray (*orf2*), and striped (*orf3*) pentagons. To generate mutants with deletions in *orf1*, *orf2*, and *orf3*, two sets of DNA fragments were amplified with the appropriate primers and ligated with artificial *Bam*HI sites as described in Materials and Methods. Sc, *Sac*I; Sl, *Sal*I; H, *Hind*III; B, artificial *Bam*HI.

100,000 \times g for 30 min. The resulting pellet was suspended in water and used as purified flagellin.

SDS-PAGE analysis and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (16). For detection of flagellin proteins, the bacterial pellet was solubilized in a sample buffer and then subjected to 12% acrylamide gel electrophoresis. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England) for immunological detection as previously described (27).

Detection of glycoproteins. For detection of glycoproteins, the SDS-PAGE gel was stained by using a GelCode glycoprotein staining kit (Pierce, Rockford, Ill.) as previously described (28).

NBT reduction assay. O_2^- was detected by the nitroblue tetrazolium (NBT) reduction assay as described previously (28), with a slight modification. Cells of the wild-type strain or each mutant bacterium, which had been grown in LB medium with 10 mM $MgCl_2$, were resuspended in 10 mM $MgSO_4$ at a concentration of 1×10^9 CFU ml^{-1} , and they were used as a bacterial suspension. Two-milliliter aliquots of a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 2.5 mg of NBT (Wako Pure Chemical Inc., Osaka, Japan) ml^{-1} , and 0.1 volume of the bacterial suspension were poured into six-well plates. Tobacco leaf disks were floated on the reaction mixture with the abaxial leaf surface down and incubated at 25°C with gentle agitation. During this incubation, O_2^- generation was monitored by determining the concentration of blue formazan converted from NBT at 560 nm.

Sequencing. For DNA sequencing, DNA fragments of the flagellum gene and the glycosylation island were subcloned into plasmids as shown in Fig. 1A. DNA sequencing was performed with a DNA sequencer (ABI PRISM TM3100; PE Applied Biosystems, Chiba, Japan) by using a BIG Dye terminator cycle sequencing kit. Amino acid sequence similarities were investigated by using the Blast search protocol and Genetyx-Mac (Software Development, Tokyo, Japan).

Nucleotide sequence accession number. The nucleotide sequence of the flagellum gene cluster with the glycosylation island has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB106910.

RESULTS

Structural analysis of putative glycosylation island. To obtain the genomic sequence of the region surrounding a *fliC* gene of *P. syringae* pv. *glycinea*, we screened a genomic clone from a DNA library. One phage clone containing an *fliC* gene was isolated by using the PCR product of the *fliC* gene (27) as a DNA probe, and then the nucleotide sequence of a 15.3-kb region of the phage clone was determined. The sequence data revealed three ORFs, designated *orf1*, *orf2*, and *orf3*, between the flagellum structural genes *flgL* and *fliC*. These three ORFs are thought to be transcribed in the same direction as the *fliC* gene (Fig. 1). The spacer regions between *flgL* and *orf1*, between *orf1* and *orf2*, and between *orf2* and *orf3* are 88, 141, and 283 bp long, respectively. We searched for promoter sequences in the spacer regions by using Genetyx-Mac and found that each of these ORFs has its own promoter.

The annotation of these ORFs is summarized in Table 2. The predicted numbers of amino acids encoded and molecular masses are 1,191 amino acids and 134 kDa for *orf1*, 968 amino acids and 107 kDa for *orf2*, and 308 amino acids and 33 kDa for *orf3*, respectively. *orf1* and *orf2* are homologous to each other, and there is 27.9% amino acid identity between the ca. 660-amino-acid regions encoded by the central region of *orf1* and the C terminus of *orf2*. Recently, the whole genome sequence of *P. syringae* pv. tomato strain DC3000 was determined (<http://www.ncbi.nlm.nih.gov/genomes/framiki.cgi?db=genome&gi=279>). The genetic region between *flgL* and *fliC* in DC3000

TABLE 2. Similarities of *P. syringae* pv. *glycinea* ORFs to proteins in databases

Gene	Size of product (amino acids)	Homologue			
		Protein and function	Bacterium	% Amino acid homology	Accession no. (reference)
<i>orf1</i>	1,191	Putative glycosyltransferase	<i>P. syringae</i> pv. tomato	92.9	AE016862-221 (unpublished data)
		OrfN, putative glycosyltransferase	<i>P. aeruginosa</i>	31.7	AF332547 (1)
		RfbC, O-antigen biosynthesis	<i>Myxococcus xanthus</i>	30.1	U36795 (11)
		Y4gl, biosynthetic protein for rhamnose-rich lipopolysaccharide	<i>Rhizobium</i> sp. strain NGR234	30.3	AE000074 (8)
<i>orf2</i>	968	Putative glycosyltransferase	<i>P. syringae</i> pv. tomato	91.6	AE016862-222 (unpublished data)
		OrfN, putative glycosyltransferase	<i>P. aeruginosa</i>	37.8	AF332547 (1)
		RfbC, O-antigen biosynthesis	<i>Myxococcus xanthus</i>	29.9	U36795 (11)
		Y4gl, biosynthetic protein for rhamnose-rich lipopolysaccharide	<i>Rhizobium</i> sp. strain NGR234	28.3	AE000074 (8)
<i>orf3</i>	308	Putative 3-oxoacyl-(acyl carrier protein) synthase III (partial sequence)	<i>P. syringae</i> pv. tabaci	100	AB061230-1 (24)
		Putative 3-oxoacyl-(acyl carrier protein) synthase III	<i>P. syringae</i> pv. tomato	98.7	AE016862-223 (unpublished data)
		Putative 3-oxoacyl-(acyl carrier protein) synthase III	<i>P. putida</i> KT2440	82.7	AE016790-134 (19)
		OrfC, unknown	<i>P. aeruginosa</i>	29.2	AF332547 (1)

contains three corresponding ORFs, *orf1*, *orf2*, and *orf3*. Each ORF in DC3000 exhibited the highest level of homology to the corresponding ORF in *P. syringae* pv. *glycinea* (the sequences encoded by *orf1*, *orf2*, and *orf3* had 92.9, 91.6, and 98.7% homology, respectively, at the amino acid level). We also found a sequence homologous to *orf3* of *P. syringae* pv. tomato DC3000 and *P. syringae* pv. *glycinea* in the upstream region of *fliC* of *P. syringae* pv. tabaci (24), suggesting that in *P. syringae* these ORFs are conserved at the same location in the genome.

orf1 and *orf2* also showed significant homology to *orfN*, which was found in the glycosylation island of *P. aeruginosa* strain PAK; at the amino acid level there was 31.7% homology in an 860-amino-acid region and there was 37.8% homology in a 577-amino-acid region, respectively (1). The proteins encoded by *orf1* and *orf2* also exhibited homology to RfbC, an O-antigen biosynthesis protein of *Myxococcus xanthus* (30% homology) (11), and y4gl, a biosynthetic protein for rhamnose-rich lipopolysaccharide in the symbiotic plasmid of *Rhizobium* sp. strain NGR234 (28 to 30% homology) (8). These levels of homology suggest that *orf1* and *orf2* encode glycosyltransferase proteins. The *orf1* and *orf2* products also are similar to the putative protein RbfC of *Xanthomonas campestris* pv. *campestris* strain ATCC 33913 (37.3 and 43.5% homology, respectively) (6; data not shown) and a hypothetical protein (PA1091) of *P. aeruginosa* strain PAO1 (42.5 and 34.1% homology, respectively) (26; data not shown). The functions of these hypothetical proteins of *X. campestris* pv. *campestris* and *P. aeruginosa* are not clear yet. On the other hand, the *orf3* product showed significant homology to putative 3-oxoacyl-(acyl carrier protein) synthase III of *Pseudomonas putida* strain KT2440 (82.7% homology) (19) and the product of *orfC* in the glycosylation island of *P. aeruginosa* strain PAO1 (29.2% homology) (1).

Generation of $\Delta orf1$, $\Delta orf2$, and $\Delta orf3$ mutants of *P. syringae* pv. *glycinea*. To examine whether the genes that exhibited homology to the genes encoding putative glycosyltransferases are responsible for flagellin glycosylation in *P. syringae* pv.

glycinea, we generated a defective mutant with a mutation in each ORF based on the homologous recombination method. To analyze the effect of the mutation in each ORF on the molecular masses of the flagellin proteins, we performed a Western blot analysis using whole proteins of the resulting mutants with anti-flagellin antibody (Fig. 2A). Western blot analysis showed that the flagellin protein of the $\Delta orf1$ mutant migrated at a position that was approximately 3 kDa lower than the position of the protein of the wild-type strain. These mobility data revealed that there was a ca. 29-kDa protein, which is consistent with the predicted sizes of the protein deduced from the sequence of the *fliC* gene and deglycosylated flagellin as previously reported (28). The molecular mass of the flagellin protein in the $\Delta orf2$ mutant was also less than the molecular mass of the flagellin protein in the wild-type strain,

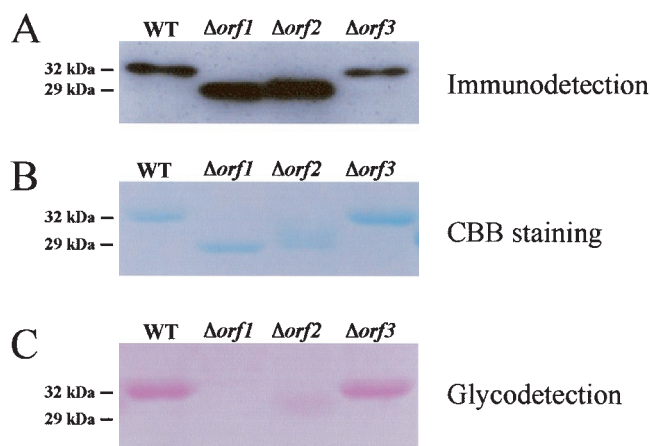


FIG. 2. Glycosylation of flagellins from the wild type (WT) and the $\Delta orf1$, $\Delta orf2$, and $\Delta orf3$ mutants of *P. syringae* pv. *glycinea*. (A) Western blot analysis of the flagellin protein in the total proteins. (B) Coomassie brilliant blue (CBB) staining of the purified flagellins. (C) Staining of the glycosyl moiety in purified flagellin.

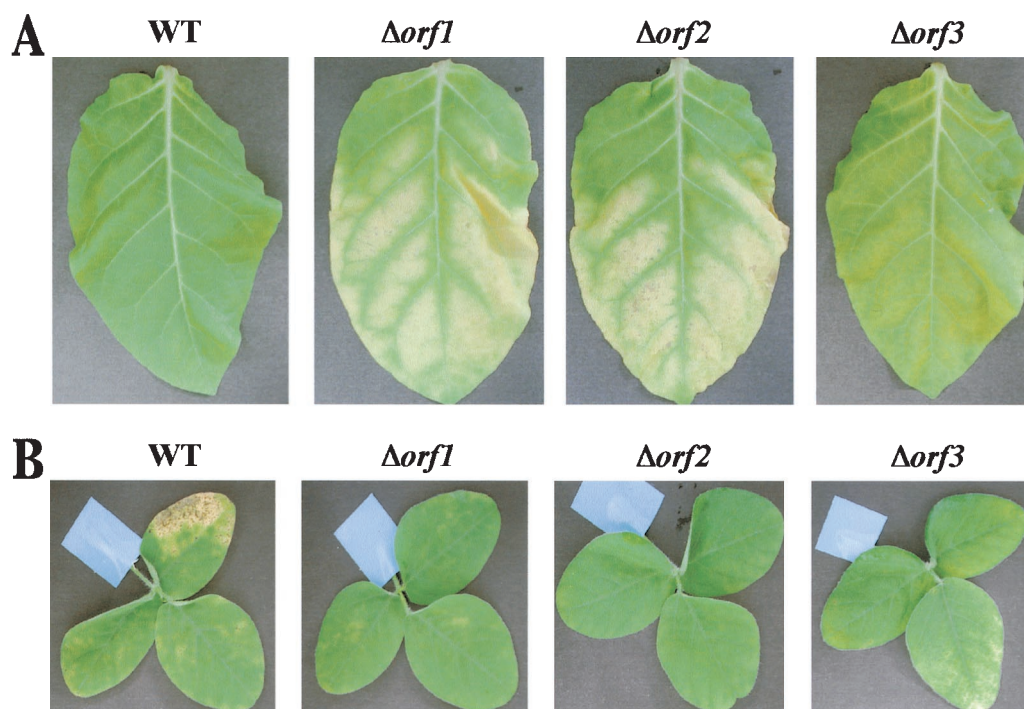


FIG. 3. Inoculation tests with nonhost tobacco leaves (A) and host soybean leaves (B). Leaves were inoculated with each bacterium by spraying. Tobacco and soybean leaves were photographed 10 and 13 days after inoculation, respectively. The tests were repeated two or three times. The photographs show typical results. WT, wild type.

but it was a little more than the molecular mass of the flagellin protein in the $\Delta orf1$ mutant. The effect of deletion of *orf3* was not obvious in the mobility of flagellin, as determined by the Western blot analysis. We also analyzed the molecular masses of purified flagellins from wild-type and mutant strains of *P. syringae* pv. *glycinea* by SDS-PAGE. As shown in Fig. 2B, the mobilities of purified flagellins were very consistent with the results obtained by Western blot analysis.

The glycosylation status of purified flagellin proteins from each mutant was examined by using a GelCode glycoprotein staining kit (Pierce). As shown in Fig. 2C, glycosylation was not detected in flagellin from the $\Delta orf1$ mutant, and only a small amount of glycosylation was detected in flagellin from the $\Delta orf2$ mutant. There was no difference in the intensities of glycodetection for flagellins from the wild-type strain and the $\Delta orf3$ mutant, suggesting that *orf3* has little, if any, responsibility for glycosylation of flagellin and the mobility shift in molecular mass.

Furthermore, deletion of each ORF had no effect on the motility on a soft agar plate, as tested by a swarming assay. It also had no effect on the doubling time (data not shown).

Inoculation of mutant bacteria onto tobacco and soybean leaves. To evaluate the effect of a defect in the genes in the glycosylation island on host and nonhost plants, we inoculated each bacterium onto tobacco (nonhost) and soybean (host) leaves (Fig. 3 and 4). For the symptom assay, plants were spray inoculated as described in Materials and Methods. Inoculation of the nonhost tobacco leaves with the $\Delta orf1$ and $\Delta orf2$ mutants resulted in lesion-like changes 10 days after inoculation, whereas inoculation with the wild-type strain did not have this effect (Fig. 3A). Furthermore, inoculation of the $\Delta orf3$ mutant

resulted in a phenotype of tobacco leaves intermediate between the phenotype of wild type-inoculated leaves and the phenotype of $\Delta orf1$ mutant-inoculated leaves. The symptoms caused by the three mutants differed in degree and speed. Tobacco leaves inoculated with the $\Delta orf1$ mutant exhibited the most drastic changes compared to the leaves inoculated with the wild-type strain. In contrast, when host soybean leaves were inoculated with each strain of *P. syringae* pv. *glycinea*, the wild-type strain caused symptoms 13 days after inoculation. However, the $\Delta orf1$ and $\Delta orf2$ mutants failed to cause prominent symptoms, and the $\Delta orf3$ mutant caused only weak symptoms (Fig. 3B).

To examine whether the disease symptoms shown in Fig. 3 were accompanied by bacterial propagation, the bacterial growth in each inoculated leaf was analyzed (Fig. 4). In tobacco leaves, all bacteria could transiently propagate within 1 day; however, by 2 days after inoculation, the population of wild-type *P. syringae* pv. *glycinea* had decreased remarkably, while the populations of the three mutants remained high (Fig. 4A). On the other hand, we observed propagation of wild-type *P. syringae* pv. *glycinea* in soybean leaves, whereas the populations of the $\Delta orf1$ and $\Delta orf2$ mutants were suppressed compared to the wild-type population. However, suppression of growth of the $\Delta orf3$ mutant was not obvious (Fig. 4B). Thus, the reduced virulence of these mutants that we observed (Fig. 3B) on the host soybean plants was reflected in the bacterial growth (Fig. 4B).

Effects of mutant bacteria on oxidative burst in nonhost tobacco leaves. Oxidative burst is known to be a typical early defense response of nonhost plants when they are inoculated with avirulent pathogens. A previous study based on an NBT

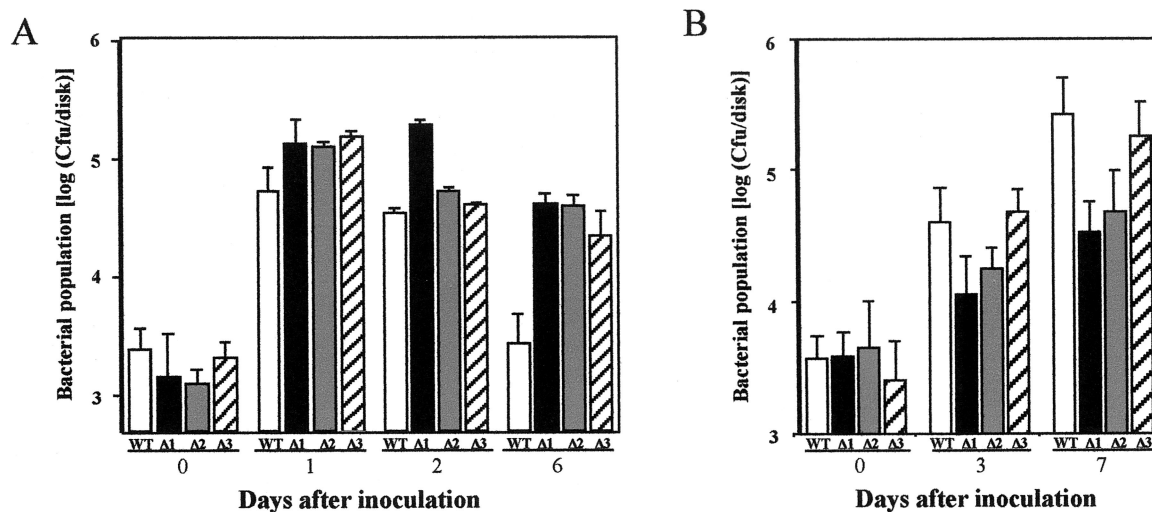


FIG. 4. Bacterial growth in tobacco leaves (A) and soybean leaves (B) inoculated with *P. syringae* pv. *glycinea* wild type (WT), the $\Delta orf1$ mutant ($\Delta 1$), the $\Delta orf2$ mutant ($\Delta 2$), or the $\Delta orf3$ mutant ($\Delta 3$). The bacterial populations in the leaves were measured 0, 1, 2, and 6 days after inoculation onto tobacco leaves and 0, 3, and 7 days after inoculation onto soybean leaves. The bars indicate means and the error bars indicate standard errors for four independent experiments.

reduction assay showed that *P. syringae* pv. *glycinea* and *P. syringae* pv. *tomato* caused prominent oxidative bursts when they were inoculated onto nonhost tobacco leaves, whereas *P. syringae* pv. *tabaci*, a bacterium with virulence for tobacco, did not (28). To evaluate the effect of deleting each ORF on the ability to cause oxidative burst in tobacco, O_2^- generation was monitored by the NBT reduction assay. As shown in Fig. 5, the levels of O_2^- generation caused by the mutants, especially the

$\Delta orf1$ and $\Delta orf2$ mutants, were lower than that caused by the wild type.

DISCUSSION

Glycosylation of flagellin is found in some pathogenic bacteria, such as *P. aeruginosa* (1), *Campylobacter jejuni* (30), *Campylobacter coli* (17), and *Helicobacter pylori* (14), but its influence on pathogenicity is poorly understood, especially in phytopathogenic bacteria. In phytopathogenic bacteria, the ability of flagellin to elicit a plant defense response was reported previously for several pathovars of *P. syringae* (7, 27, 28) and *P. avenae* (5). Che et al. (5) and Taguchi et al. (28) detected glycosylation in flagellin proteins in *P. avenae* and *P. syringae*, respectively. Taguchi et al. also suggested the importance of posttranslational modification of flagellin proteins of *P. syringae* in flagellin-induced hypersensitive death of tobacco cells (27, 28). In this study, we identified the genes involved in flagellin glycosylation and examined their influence on plant-microbe interactions.

Three ORFs, *orf1*, *orf2*, and *orf3*, located between the *flgL* and *fliC* genes, were termed the glycosylation island. The putative proteins encoded by *orf1* and *orf2* exhibited 31.7 and 37.8% similarity, respectively, to OrfN of the glycosylation island in *P. aeruginosa* strain PAK (1). Interestingly, amino acid sequences encoded by *orf1* and *orf2* exhibited 27.9% identity to each other. Deletion of *orf1* and *orf2* resulted in a reduction in the molecular mass as determined by SDS-PAGE analysis (Fig. 2A and B), indicating that both gene products are necessary for flagellin glycosylation. The deletion of *orf1* completely eliminated the ability to glycosylate flagellin proteins, whereas some glycosyl residues remained in the flagellin of the $\Delta orf2$ mutant (Fig. 2C). In the biosynthetic pathway, several glycosyltransferases are thought to be required to form one mature glycoprotein. In the system which we examined, two glycosyltransferases are thought to function in a certain order.

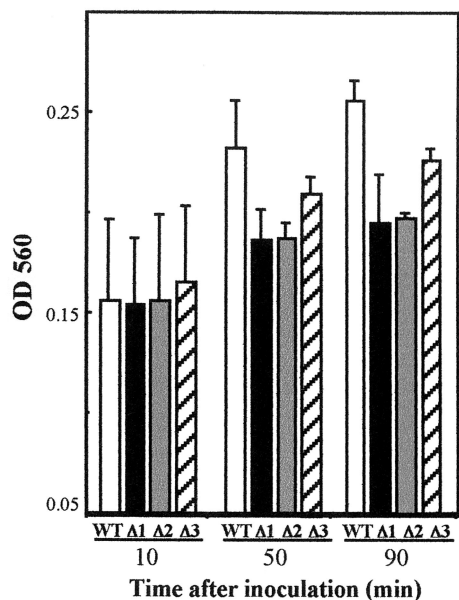


FIG. 5. NBT reduction assay on the surfaces of tobacco leaves inoculated with *P. syringae* pv. *glycinea* wild type (WT), the $\Delta orf1$ mutant ($\Delta 1$), the $\Delta orf2$ mutant ($\Delta 2$), or the $\Delta orf3$ mutant ($\Delta 3$). Formation of blue formazan was measured 10, 50, and 90 min after inoculation. The bars indicate the means and the error bars indicate the standard errors for four independent experiments. OD 560, optical density at 560 nm.

Our results indicate that the *orf1* product seemed initially to transfer some saccharide(s) to flagellin proteins, and this was followed by modification by *orf2*. The Δ *orf1* and Δ *orf2* mutants acquired pathogenicity to originally nonhost tobacco plants (Fig. 3A and 4A). In other words, the Δ *orf1* and Δ *orf2* mutants have reduced abilities to induce an oxidative burst in tobacco (Fig. 5), which allows parasitic propagation of bacteria and expansion of the disease symptoms. Thus, glycosyl moieties of flagellins from incompatible pathogenic bacteria seem to possess the ability to induce a plant defense response. In the Δ *orf3* mutant, the molecular mass of flagellin was not obviously changed, as determined by SDS-PAGE analysis. However, an inoculation test with nonhost tobacco leaves revealed that the growth of the Δ *orf3* mutant was greater than that of the wild type (Fig. 4A). Furthermore, the level of oxidative burst caused by the Δ *orf3* mutant was slightly lower than that caused by the wild type. Thus, *orf3* is also likely to contribute to formation of an epitope for the HR-inducing elicitor. Orf3 showed significant homology to 3-oxoacyl-(acyl carrier protein) synthase III. The role of *orf3* in flagellin modification is obscure at present, but considering that acylation of lipooligosaccharides has been reported to be involved in the sensitivity of nontypeable *Haemophilus influenzae* to human β -defensins (25), acylation of flagellin seems to have a considerable role in innate immunity.

Because the Δ *orf1* and Δ *orf2* mutants failed to invade the original host soybean plants, addition or variation of the glycosyl moiety may reflect an evolutionary adaptation of flagellin to avoid recognition by the host defense surveillance system. In many cases, a carbohydrate modification rather than amino acid epitopes appears to represent an immune-dominant determinant (4). A previous study showed that flagellin glycosylation only in the manner found in *P. syringae* pv. *tabaci* eliminated HR death of tobacco cells (28). Thus, to establish a compatible relationship, flagellin proteins need to be glycosylated in the original pathogenic bacterial manner. In the case of *P. syringae* pv. *glycinea*, the glycosyl moiety of flagellin might interfere with efficient recognition by host soybean cells, leading to HR death of cells, and this may be the reason that *P. syringae* pv. *glycinea* has the ability to invade soybean plants. Further investigation of this is necessary.

Why do flagellin proteins need to be glycosylated? Although the results of the swarming assay showed that all mutants retain mobility, we speculate that the glycosylation of flagellin might increase the hydrophilicity of flagella. When incompatible relationships are considered, plants might have evolved by acquisition of the ability to recognize flagellin, especially its glycosyl moieties, and to initiate the defense responses. In general, race-cultivar specificity in plant-pathogen interactions is explained by the ligand-receptor model based on the gene-for-gene theory of plant disease resistance (23, 29). In this model, an avirulence protein encoded by a bacterial avirulence (*avr*) gene is considered a ligand that is recognized by a corresponding receptor encoded by a plant resistance (*R*) gene. Once a plant recognizes avirulent bacteria, the HR, involving rapid plant cell death at the point of invasion, occurs. Although the products recognized by the tobacco plant are supposedly not putative glycosyltransferases themselves, *orf1* and *orf2* should be defined as *avr*-like genes in the sense that these genes are thought to endow *P. syringae* pv. *glycinea* with avirulence for tobacco. An interesting example of an indirect aviru-

lence gene is *avrD* of *P. syringae* pv. *tomato* (18). *avrD* specifies production of a low-molecular-weight compound, syringolide, that triggers plant defense responses via the condensation of D-xylulose and a β -ketoalkanoic acid. Recently, Boller and colleagues found that the *FLS2* gene, encoding a leucine-rich repeat receptor-like kinase, is involved in recognition of *flg22* and leads to activation of defense responses (2, 3, 9). *flg22* has not been reported to induce HR cell death (10). On the other hand, flagellin proteins purified from each pathovar have been found to elicit HR in nonhost plants (27). Thus, the flagellin molecule is thought to possess at least two epitopes that trigger plant defense responses; one is a region that corresponds to *flg22* and induces general defense responses without cell death, and the other is a peptide region outside *flg22* and/or the glycosylation motif in flagellin that induces HR cell death. Further investigation is required to determine the role of flagellin glycosylation in plant-pathogen interactions.

The glycosyl structures and their amino acid linkages that are responsible for the observed changes in virulence and avirulence remain to be determined. The lengths of the sugar chains in pathogenic bacterial proteins so far are considered shorter (mono- to trisaccharide substituents) than those of eukaryotes (4). If this idea is applicable to our study, about 10 sugar chains would be attached to the flagellin peptide region by a 2- to 3-kDa shift in the SDS-PAGE assay (Fig. 2). We identified only two genes for putative glycosyltransferases; thus, the structure of carbohydrates linked to flagellin might not be very complicated. Otherwise, these glycosyltransferases might be multicatalytic enzymes, because some of the bacterial glycosyltransferases involved in polysaccharide synthesis have been reported to be bifunctional (21, 31), and the predicted molecular masses of both *orf1* and *orf2* are relatively large (134 kDa for *orf1* and 107 kDa for *orf2*). However, we cannot exclude the possibility that there may be other glycosyltransferases that are responsible for flagellin glycosylation.

Recently, a sequence that is highly homologous to the glycosylation island in *P. syringae* pv. *tabaci* was found in the upstream region of the *fliC* gene (24; F. Taguchi, K. Takeuchi, H. Kaku, E. Katoh, K. Murata, T. Kawasaki, Y. Inagaki, K. Toyoda, T. Shiraiishi, and Y. Ichinose, unpublished data), which suggests that glycosyltransferases are present. It is conceivable that these regions of the two pathovars determine the difference in the flagellins characterized as ligands for plant cells. We are now investigating the role of the glycosylation of flagellin in the specificity of pathovars for plant species by swapping the glycosylation islands in *P. syringae* pv. *glycinea* and *P. syringae* pv. *tabaci*. The molecular mechanisms of plant recognition might be clarified by utilizing the mutant strains that have been produced.

ACKNOWLEDGMENTS

We thank A. Collmer (Cornell University, Ithaca, N.Y.) for providing *P. syringae* pv. *glycinea* and Chihiro Yasuda for technical assistance with the inoculation experiments. We are also grateful to T. Mukaihara (RIBS, Okayama, Japan) and Y. Kimura (Okayama University, Okayama, Japan) for valuable discussions concerning the generation of mutant bacteria and glycosylation studies, respectively.

This work was supported in part by Grant-in-Aids for Scientific Research on Priority Area (A) 12052215 and 12052219 and for Scientific Research (S) 15108001 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

- Arora, S. K., M. Banger, S. Lory, and R. Ramphal. 2001. A genomic island in *Pseudomonas aeruginosa* carries the determinants of flagellin glycosylation. *Proc. Natl. Acad. Sci. USA* **98**:9342–9347.
- Asai, T., G. Tena, J. Plotnikova, M. R. Willmann, W. L. Chiu, L. Gomez-Gomez, T. Boller, F. M. Ausubel, and J. Sheen. 2002. MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* **415**:977–983.
- Bauer, Z., L. Gomez-Gomez, T. Boller, and G. Felix. 2001. Sensitivity of different ecotypes and mutants of *Arabidopsis thaliana* towards the bacterial elicitor flagellin correlates with the presence of receptor binding sites. *J. Biol. Chem.* **276**:45669–45676.
- Benz, I., and M. A. Schmidt. 2002. Never say never again: protein glycosylation in pathogenic bacteria. *Mol. Microbiol.* **45**:267–276.
- Che, F. S., Y. Nakajima, N. Tanaka, M. Iwano, T. Yoshida, S. Takayama, I. Kadota, and A. Isogai. 2000. Flagellin from an incompatible strain of *Pseudomonas avenae* induces a resistance response in cultured rice cells. *J. Biol. Chem.* **275**:32347–32356.
- da Silva, A. C., J. A. Ferro, F. C. Reinach, C. S. Farah, L. R. Furlan, R. B. Quaggio, C. B. Monteiro-Vitorello, M. A. Van Sluys, N. F. Almeida, L. M. Alves, A. M. do Amaral, M. C. Bertolini, L. E. Camargo, G. Camarotte, F. Cannavan, J. Cardozo, F. Chambergo, L. P. Ciapina, R. M. Cicarelli, L. L. Coutinho, J. R. Cursino-Santos, H. El-Dorry, J. B. Faria, A. J. Ferreira, R. C. Ferreira, M. I. Ferro, E. F. Formighieri, M. C. Franco, C. C. Greggio, A. Gruber, A. M. Katsuyama, L. T. Kishi, R. P. Leite, E. G. Lemos, M. V. Lemos, E. C. Locali, M. A. Machado, A. M. Madeira, N. M. Martinez-Rossi, E. C. Martins, J. Meidanis, C. F. Menck, C. Y. Miyaki, D. H. Moon, L. M. Moreira, M. T. Novo, V. K. Okura, M. C. Oliveira, V. R. Oliveira, H. A. Pereira, A. Rossi, J. A. Sena, C. Silva, R. F. de Souza, L. A. Spinola, M. A. Takita, R. E. Tamura, E. C. Teixeira, R. I. Tezza, M. Trindade dos Santos, D. Truffi, S. M. Tsai, F. F. White, J. C. Setubal, and J. P. Kitajima. 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature* **417**:459–463.
- Felix, G., J. D. Duran, S. Volko, and T. Boller. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **18**:265–276.
- Freiberg, C., R. Fellay, A. Bairoch, W. J. Broughton, A. Rosenthal, and X. Perret. 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. *Nature* **387**:394–401.
- Gomez-Gomez, L., Z. Bauer, and T. Boller. 2001. Both the extracellular leucine-rich repeat domain and the kinase activity of FSL2 are required for flagellin binding and signaling in *Arabidopsis*. *Plant Cell* **13**:1155–1163.
- Gomez-Gomez, L., and T. Boller. 2002. Flagellin perception: a paradigm for innate immunity. *Trends Plant Sci.* **7**:251–256.
- Guo, D., M. G. Bowden, R. Pershad, and H. B. Kaplan. 1996. The *Myxococcus xanthus* *rjbABC* operon encodes an ATP-binding cassette transporter homolog required for O-antigen biosynthesis and multicellular development. *J. Bacteriol.* **178**:1631–1639.
- Huynh, T., D. Dahlbeck, and B. Staskawicz. 1989. Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science* **245**:1374–1377.
- Ichinose, Y., R. Shimizu, Y. Ikeda, F. Taguchi, M. Marutani, T. Mukaihara, Y. Inagaki, K. Toyoda, and T. Shiraishi. 2003. Need for flagella for complete virulence of *Pseudomonas syringae* pv. *tabaci*: genetical analysis with flagella defective mutants, Δ *flhC* and Δ *flhD*, in host tobacco plants. *J. Gen. Plant Pathol.* **69**:244–249.
- Josenhans, C., L. Vossebein, S. Friedrich, and S. Suerbaum. 2002. The *neuA/flmD* gene cluster of *Helicobacter pylori* is involved in flagellar biosynthesis and flagellin glycosylation. *FEMS Microbiol. Lett.* **210**:165–172.
- King, E. O., N. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyrocyanine and fluorescein. *J. Lab. Clin. Med.* **44**:301–307.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Logan, S. M., J. F. Kelly, P. Thibault, C. P. Ewing, and P. Guerry. 2002. Structural heterogeneity of carbohydrate modifications affects serospecificity of *Campylobacter* flagellins. *Mol. Microbiol.* **46**:587–597.
- Midland, S. L., N. T. Keen, and J. J. Sims. 1993. The structures of syringolides 1 and 2, novel C-glycosidic elicitors from *Pseudomonas syringae* pv. *tomato*. *J. Org. Chem.* **58**:2940–2945.
- Nelson, K. E., C. Weinel, I. T. Paulsen, R. J. Dodson, H. Hilbert, V. A. Martins dos Santos, D. E. Fouts, S. R. Gill, M. Pop, M. Holmes, L. Brinkac, M. Beanan, R. T. DeBoy, S. Daugherty, J. Kolonay, R. Madupu, W. Nelson, O. White, J. Peterson, H. Khouri, I. Hance, P. Cris Lee, E. Holtzapple, D. Scanlan, K. Tran, A. Moazzez, T. Utterback, M. Rizzo, K. Lee, D. Kosack, D. Moestl, H. Wedler, J. Lauber, D. Stjepandic, J. Hoheisel, M. Straetz, S. Heim, C. Kiewitz, J. Eisen, K. N. Timmis, A. Dusterhoft, B. Tummeler, and C. M. Fraser. 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.* **4**:799–808.
- Power, P. M., and M. P. Jennings. 2003. The genetics of glycosylation in Gram-negative bacteria. *FEMS Microbiol. Lett.* **218**:211–222.
- Rocchetta, H. L., L. L. Burrows, J. C. Pacan, and J. S. Lam. 1998. Three rhamnosyltransferases responsible for assembly of the A-band D-rhamnan polysaccharide in *Pseudomonas aeruginosa*: a fourth transferase, WbpL, is required for the initiation of both A-band and B-band lipopolysaccharide synthesis. *Mol. Microbiol.* **28**:1103–1119.
- Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, and A. Pühler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**:69–73.
- Scofield, S. R., C. M. Tobias, J. P. Rathjen, J. H. Chang, D. T. Lavelle, R. W. Michelmore, and B. J. Staskawicz. 1996. Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science* **274**:2063–2065.
- Shimizu, R., F. Taguchi, M. Marutani, T. Mukaihara, Y. Inagaki, K. Toyoda, T. Shiraishi, and Y. Ichinose. 2003. The Δ *flhD* mutant of *Pseudomonas syringae* pv. *tabaci*, which secretes flagellin monomers, induces a strong hypersensitive reaction (HR) in non-host tomato cells. *Mol. Genet. Genomics* **269**:21–30.
- Starner, T. D., W. E. Swords, M. A. Apicella, and P. B. McCray, Jr. 2002. Susceptibility of nontypeable *Haemophilus influenzae* to human beta-defensins is influenced by lipooligosaccharide acylation. *Infect. Immun.* **70**:5287–5289.
- Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrenner, M. J. Hickey, F. S. L. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrook-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K.-S. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. W. Hancock, S. Lory, and M. V. Olson. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**:959–964.
- Taguchi, F., R. Shimizu, R. Nakajima, K. Toyoda, T. Shiraishi, and Y. Ichinose. 2003. Differential effects of flagellins from *Pseudomonas syringae* pvs. *tabaci*, *tomato* and *glycinea* on plant defense response. *Plant Physiol. Biochem.* **41**:165–174.
- Taguchi, F., R. Shimizu, Y. Ikeda, Y. Inagaki, K. Toyoda, T. Shiraishi, and Y. Ichinose. 2003. Post-translational modification of flagellin determines the specific induction of HR. *Plant Cell Physiol.* **44**:342–349.
- Tang, X. Y., R. D. Frederick, J. M. Zhou, D. A. Halterman, Y. L. Jia, and G. B. Martin. 1996. Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science* **274**:2060–2063.
- Thibault, P., S. M. Logan, J. F. Kelly, J.-R. Brisson, C. P. Ewing, T. J. Trust, and P. Guerry. 2001. Identification of the carbohydrate moieties and glycosylation motifs in *Campylobacter jejuni* flagellin. *J. Biol. Chem.* **276**:34862–34870.
- Zhang, L., J. Radziejewska-Lebrecht, D. Krajewska-Pietrasik, P. Toivanen, and M. Skurnik. 1997. Molecular and chemical characterization of the lipopolysaccharide O-antigen and its role in the virulence of *Yersinia enterocolitica* serotype O:8. *Mol. Microbiol.* **23**:63–76.