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# *Burkholderia cenocepacia* Lipopolysaccharide Modification and Flagellin Glycosylation Affect Virulence but Not Innate Immune Recognition in Plants

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**ABSTRACT** *Burkholderia cenocepacia* causes opportunistic infections in plants, insects, animals, and humans, suggesting that “virulence” depends on the host and its innate susceptibility to infection. We hypothesized that modifications in key bacterial molecules recognized by the innate immune system modulate host responses to *B. cenocepacia*. Indeed, modification of lipopolysaccharide (LPS) with 4-amino-4-deoxy-L-arabinose and flagellin glycosylation attenuates *B. cenocepacia* infection in *Arabidopsis thaliana* and *Galleria mellonella* insect larvae. However, *B. cenocepacia* LPS and flagellin triggered rapid bursts of nitric oxide and reactive oxygen species in *A. thaliana* leading to activation of the *PR-1* defense gene. These responses were drastically reduced in plants with *fls2* (flagellin FLS2 host receptor kinase), *Atnoa1* (nitric oxide-associated protein 1), and *dnd1-1* (reduced production of nitric oxide) null mutations. Together, our results indicate that LPS modification and flagellin glycosylation do not affect recognition by plant receptors but are required for bacteria to establish overt infection.

**IMPORTANCE** Virulence and pathogenicity are properties ascribed to microbes, which actually require careful consideration of the host. Using the term “pathogen” to define a microbe without considering its host has recently been debated, since the microbe’s capacity to establish a niche in a given host is a critical feature associated with infection. Opportunistic bacteria are a perfect example of microbes whose ability to cause disease is intimately related to the host’s ability to recognize and respond to the infection. Here, we use the opportunistic bacterium *Burkholderia cenocepacia* and the host plant *Arabidopsis thaliana* to investigate the role of bacterial surface molecules, namely, lipopolysaccharide and flagellin, in contributing to infection and also in eliciting a host response. We reveal that both molecules can be modified by glycosylation, and although the modifications are critical for the bacteria to establish an infection, they do not impact the host’s ability to recognize the pathogen.

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**B***Burkholderia cepacia* complex (BCC) denotes a group of ubiquitous Gram-negative bacterial species isolated from water, soil, plants, insects, industrial settings, and hospital environments (1). Some BCC strains have beneficial traits, as they utilize complex carbon sources to degrade toxic compounds in pesticides and herbicides, while others serve as bioremediation agents (2). However, BCC strains are opportunistic pathogens causing serious infections in immunocompromised humans, including chronic lung infection in cystic fibrosis (CF) patients (3). The most common BCC clinical isolates are *B. cenocepacia* and *B. multivorans*. In particular, *B. cenocepacia* has gained notoriety for its ability to cause lethal necrotizing pneumonia and its transmissibility among CF patients (3).

The opportunistic nature of infections by *B. cenocepacia* makes it difficult to study the pathogenic mechanisms of this bacterium, since “virulence” depends on the host’s context and its innate susceptibility to infection. Several infection models for *B. cenocepacia* have been established, which include mice

and rats (4), the zebrafish embryo (5), the nematode *Caenorhabditis elegans* (6), *Galleria mellonella* moth larvae (7, 8), and plants like alfalfa (4) and duckweed (9). Plants have evolved sophisticated mechanisms to perceive and respond to nearly constant attacks from potential pathogens (10). These mechanisms involve passive protection, such as that provided by the waxy cuticular skin layer, and innate immune responses. Like the innate immune system in mammals and insects, the plant immune system also perceives and responds to various elicitor molecules, which are conserved microbial structures referred to as microbe- or pathogen-associated molecular patterns (PAMPs). These include bacterial flagellin, lipopolysaccharide (LPS), and lipoteichoic acid and fungal chitin and ergosterol (11). LPS consists of lipid A, embedded in the outer leaflet of the outer membrane, which is linked to core oligosaccharide and polymeric O antigen (12), while flagellin is the building block of the flagellar filament essential for bacterial motility. LPS and flagellin are typical PAMPs that stimulate plant cells and tissues to generate reactive oxygen species (ROS), alkalinization of the extracel-

lular medium, callose deposition, and nitric oxide (NO) burst (13–15). These responses lead to programmed cell death localized to the tissue surrounding the infection site, which is known as the hypersensitive response (11, 16).

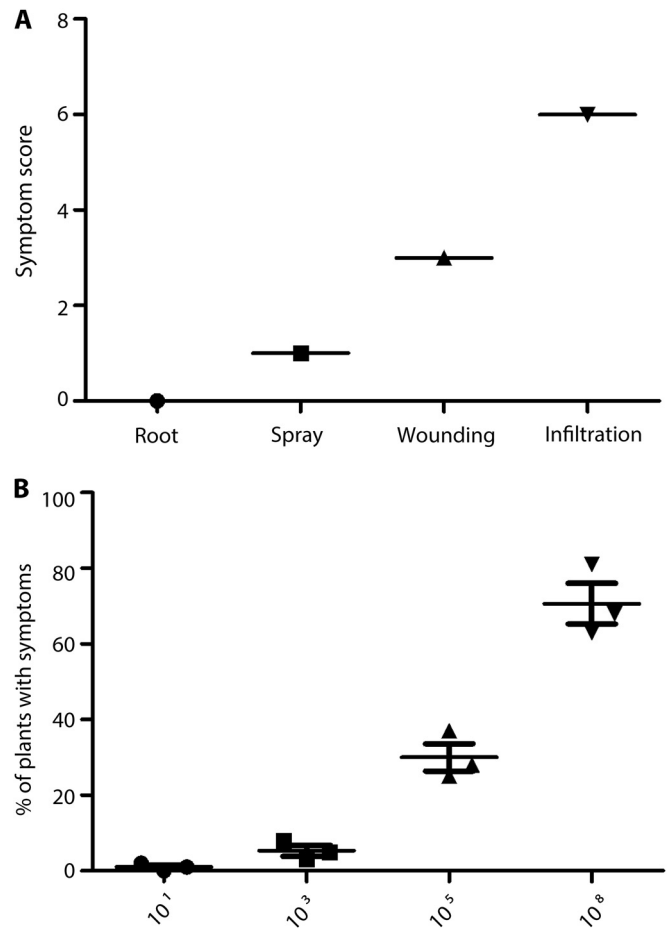
*Arabidopsis thaliana* is an established model organism for plant biology, but it is also a tool to understand molecular mechanisms of human diseases (17, 18), particularly those concerning the initial stages of infection (10, 19). Previous work by others reported a rapid burst of NO after treatment of *A. thaliana* cultured cells and leaves with *B. cenocepacia* lipid A preparations, which was associated with the transcriptional activation of defense-related genes (20). Further, treatment with *B. cenocepacia* purified lipid A and core oligosaccharide LPS components resulted in different plant transcriptional responses (21), suggesting that plant cells perceive LPS components differently. Lipid A structural modifications alter recognition and responses by the innate immune system in mammals (22) and in plants (23). LPS contributes to reduce the permeability of the outer membrane of Gram-negative bacteria, acting as a barrier for plant-derived antimicrobial substances (24). *B. cenocepacia* exhibits extraordinary intrinsic resistance to antimicrobial peptides and other antibiotics (25). We have shown that the *B. cenocepacia* lipid A and one of the sugars of the inner core oligosaccharide are modified by the incorporation of L-Ara4N, and this modification not only is essential for bacterial survival (26) but also is the major determinant of resistance to antimicrobial peptides (27). The essentiality of the LPS modification by L-Ara4N suggests the possibility that this amino sugar could also contribute to modulate innate immune responses.

Flagellin is a major activator of innate immunity in animals, where it is recognized by the Toll-like receptor 5 (TLR5) (28), and in plants (29, 30). Recognition of flagellin by plants activates disease resistance mechanisms through an oxidative burst during early stages of infection (13, 15). The *A. thaliana* flagellin sensing 2 (FLS2) protein is a PAMP receptor kinase with an extracellular domain containing 28 leucine-rich repeats, a transmembrane domain, and an intracellular serine/threonine kinase domain (31, 32). These interactions activate production of ROS, triggering a complex defense response, including induction of pathogenesis-related genes (15). We have recently shown that the *B. cenocepacia* flagellin is glycosylated, and its glycosylation status modulates innate immune responses in mammalian cells (33), but the role of flagellin glycosylation in plant immune responses to *B. cenocepacia* infection has not been examined.

In this work, we investigated the pathogenicity of *B. cenocepacia* in *A. thaliana* and evaluated the significance of LPS modification with L-Ara4N and flagellin glycosylation in virulence and in *Arabidopsis*-*B. cenocepacia* interactions by studying innate immune responses by wild-type and mutant plants. Further, we compared bacterial virulence *in planta* and in the *G. mellonella* infection model to clarify the global role of flagellin glycosylation and LPS modification on pathogenesis. We demonstrate that flagellin glycosylation and LPS modification with L-Ara4N play a significant role in bacterial survival upon infection but do not alter the perception of these molecules by the plant innate immune receptors, indicating these modifications are only critical to establish infection.

## RESULTS

**Pathogenicity of *B. cenocepacia* in *A. thaliana* and tobacco seedlings.** We investigated the ability of *B. cenocepacia* K56-2, a CF clinical isolate, to induce a hypersensitive response (HR) in



**FIG 1** HR in *A. thaliana* leaves using various inoculation methods. (A) Quantitative scoring of HR in leaves of wild-type *Arabidopsis* inoculated with K56-2 by different inoculation methods. Symptom scores were assigned using an index from 1 to 6 based on the extent of tissue collapse and color change, where 0 is no symptom, 1 is random yellow or dark spots, 2 is chlorosis over the inoculation site, 3 is chlorosis and mild tissue collapse at and around the inoculation site, 4 is more than 50% collapse at and around the inoculation site, 5 is 100% collapse at the inoculation site, and 6 is 100% collapse beyond the inoculation site, as observed in day 7 postinfection. The average score was calculated based on a minimum of 45 leaves per treatment cumulative from 3 individual repeats. (B) Effect of inoculum size on the incidence of infection. Values indicate the percentage  $\pm$  standard error (SE) of seedlings showing disease symptoms at 7 days postinfection after inoculation with doses ranging from  $10^1$  to  $10^8$  CFU; 15 plants per infection dose were tested. Data represent the average from three experiments.

*A. thaliana* and tobacco. Seedlings from both species were inoculated by different methods with  $10^1$  to  $10^8$  bacteria and examined for symptoms over 1 week. A simple infection model was developed in which leaves were wounded with a needle and then inoculated with dilute suspensions of *B. cenocepacia*. Disease was scored for every seedling on an index of 1 to 6 based on the extent of HR symptoms and tissue necrosis using a standard HR scoring system (34). Depending on the bacterial dose, infection of seedlings via wound inoculation or syringe infiltration led to necrosis (brown areas on leaves at higher doses) or chlorosis (yellowing of the leaf tissue), which were clearly visible after day 4 postinfection. In contrast, infections by soil drainage or surface spray gave few symptoms even at day 7 postinfection (Fig. 1A). There was a direct

TABLE 1 Bacterial strains used in this study

Strain	Characteristic(s)	Source or reference
K56-2	<i>B. cenocepacia</i> ET12 clone related to J2315, CF clinical isolate	BCRRC <sup>a</sup>
K56-2 $\Delta$ <i>atsR</i>	K56-2 carrying an unmarked deletion of <i>atsR</i> gene	37
MH55	K56-2, $\Delta$ <i>arnT</i> - <i>arnBC</i> <sup>+</sup> <i>lptG</i> <sub>D31H</sub> ( <i>lptG</i> <sup>S</sup> )	27
$\Delta$ BCAL0111 mutant	K56-2, unmarked deletion of <i>flmQ</i> (BCAL0111)	33
MSS25	K56-2, <i>fliC</i> ::pSM62, insertional inactivation of <i>fliC</i>	35

<sup>a</sup> BCRRC, *B. cenocepacia* Research and Referral Repository for Canadian CF Clinics.

correlation between inoculum size and HR symptoms (Fig. 1B). Similar results were observed in tobacco plants, where infection via roots or leaf spraying did not cause distinguishable symptoms in plantlets (see Fig. S1 in the supplemental material). Since several mutants in innate immunity pathways and pathogen receptors are available for *A. thaliana* ecotype Colombia Col-0, we used only seedlings from this species for the remainder of this study, which were inoculated with 10<sup>8</sup> CFU via wound inoculation or syringe infiltration.

**L-Ara4N modification of LPS and flagellin glycosylation are required to cause pathology in *A. thaliana*.** *B. cenocepacia* re-

quires L-Ara4N modification of LPS for viability and resistance to antimicrobial peptides (27). However, a suppressor mutation in the *lptG* gene, which encodes an essential protein for the export of LPS to the outer membrane, restores viability in the absence of L-Ara4N LPS modification (27). We therefore investigated if lack of L-Ara4N in the LPS of this mutant (MH55;  $\Delta$ *arnT* *lptG*<sub>D31H</sub>) (Table 1) could affect *B. cenocepacia* survival in *A. thaliana*. Infection of *A. thaliana* seedlings with the  $\Delta$ *arnT* *lptG*<sub>D31H</sub> mutant caused mild symptoms of chlorosis in 27% of inoculated plants (Fig. 2A and C); very few bacteria were recovered from leaves, compared to plants infected with the K56-2 parental strain

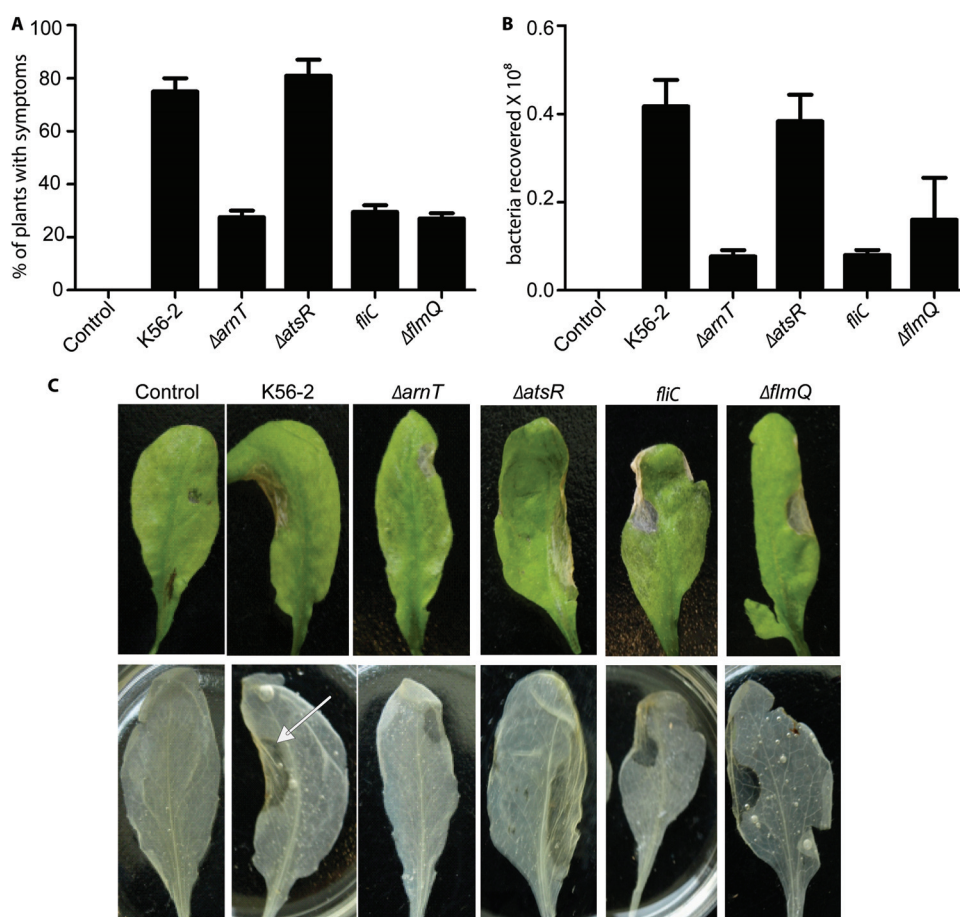
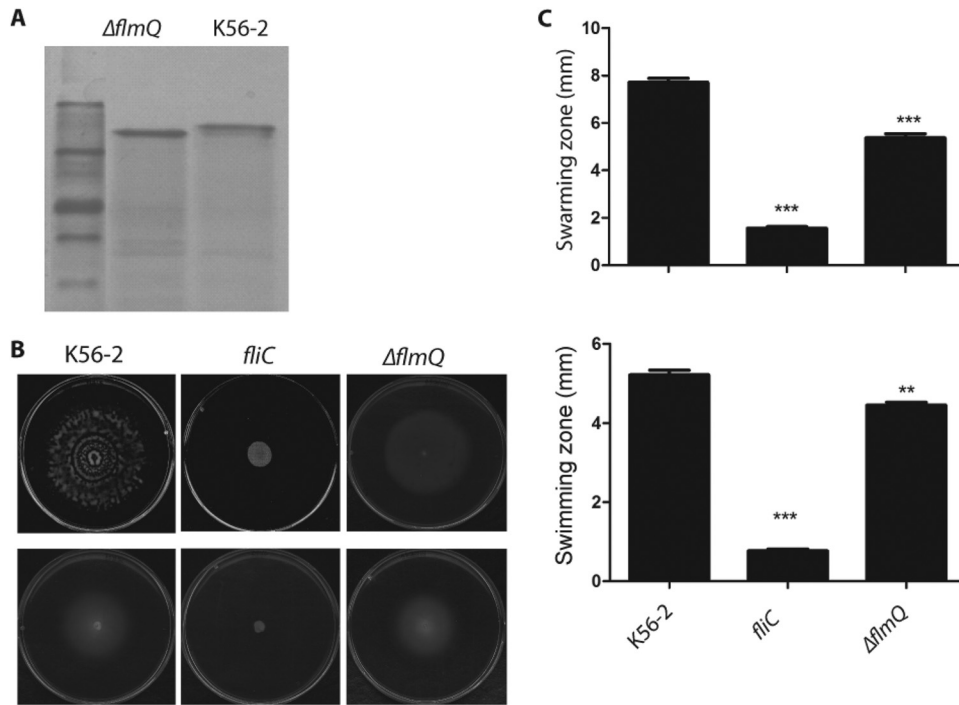


FIG 2 Infection and replication of *B. cenocepacia* strains in *Arabidopsis* seedlings. (A) Values show the percentages  $\pm$  SE of the seedlings showing disease symptoms visible on day 7 postinfection after wound inoculation with 10<sup>8</sup> CFU of each *B. cenocepacia* strain; 15 plants per infection dose were tested. Data are the average from three repeats. (B) The graph represents bacterial counts on day 7 postinfection from *Arabidopsis* leaves infected with *B. cenocepacia* by wound inoculation. (C) Photographs of representative leaves (from three replicate treatments) from *Arabidopsis* plants inoculated with *B. cenocepacia* strains by infiltration (upper row). Leaves were removed from plants and bleached in ethanol (lower row). An arrow indicates the inoculated region, which is more transparent and flattened, undergoing tissue collapse, compared with the rest of the leaf and the wild-type control treated with buffer. Similar results were observed with wound-inoculated leaves (see Fig. S4 in the supplemental material).



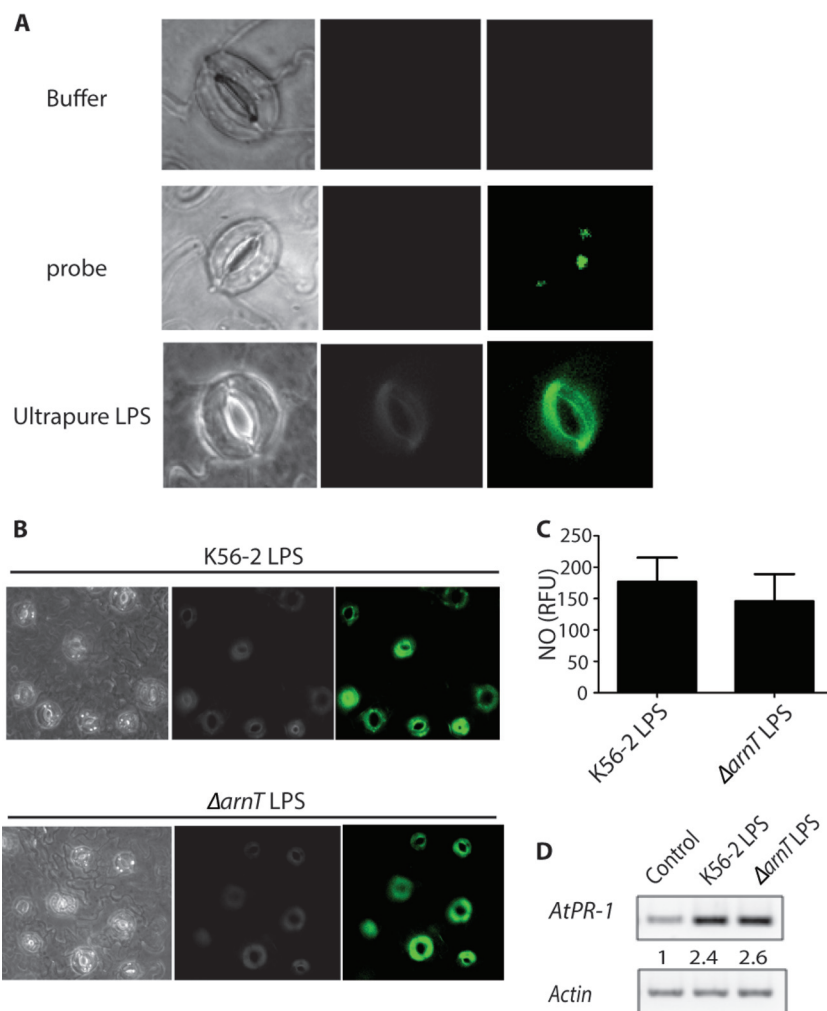
**FIG 3** Characterization of flagellin mutants. (A) SDS-PAGE analysis of purified flagellin from the WT and K56-2  $\Delta flmQ$  mutant strain of *B. cenocepacia*. (B) Swarming (upper row) and swimming (lower row) motility on soft agar plates of *B. cenocepacia* strains. The data represent three independent experiments. (C) Quantification of the motility zone. Statistical analysis was performed by paired *t* test using two-tailed *P* values. Significant differences in comparison with *B. cenocepacia* parental strain (WT) as the control are indicated by \*\*\* ( $P < 0.005$ ) and \*\* ( $P < 0.027$ ).

(Fig. 2B). These data demonstrate that L-Ara4N modification of LPS is critical for *B. cenocepacia* pathogenicity. The poor infectivity of the suppressor mutant is most likely due to the lack of L-Ara4N in the LPS of the  $\Delta arnT$   $lptG_{D31H}$  mutant, as revealed by mass spectrometry of purified lipid A (see Fig. S2 in the supplemental material), which makes it extremely sensitive to naturally produced antimicrobial peptides in plants, as we previously demonstrated with polymyxin B (27).

Next, we evaluated the significance of flagellin in *A. thaliana* pathogenicity. For these experiments, we used an *fliC* mutant that is nonmotile and unable to make flagellin (35) and the hypermotile, flagellated  $\Delta atsR$  mutant (Table 1). The *atsR* gene encodes a global regulatory repressor protein that controls motility and the expression of various virulence factors (36–38). There were no statistically significant differences in disease symptoms between plants infected with the  $\Delta atsR$  mutant and K56-2 at day 7 postinfection (Fig. 2). However, symptoms appeared 2 days earlier in  $\Delta atsR$  strain-infected plants, and these results were reproducible in each experimental replica. These observations agree with the current model for the regulatory role of AtsR (37, 38) indicating that removal of inhibitory regulation on motility as well as other AtsR-controlled factors increases bacterial pathogenicity. Therefore, it is not surprising that the  $\Delta atsR$  mutant provoked lesions similar to those found with the parental strain K56-2 (Fig. 2C). In contrast, loss of flagellin in the *fliC* mutant resulted in a strain that induced only mild HR symptoms in less than 30% of plants infected by wound inoculation (Fig. 2A), revealing the critical role of flagella in virulence and also suggesting that flagellar driven motility is important for infection. The wound inoculation method mimics entry of microbes into host plants through

natural wounds, which results in bacterial spreading to intercellular spaces. In contrast, infiltration of bacteria with a syringe into the apoplast could bypass the first steps of the natural infection process and, therefore, the requirement for bacterial motility. To probe this notion, we infected *Arabidopsis* seedlings by syringe infiltration into leaf abaxial surfaces. Under these conditions, the *fliC* mutant elicited similar HR symptoms to those induced by parental K56-2 (Fig. 2C). HR also correlated with higher numbers of bacteria recovered from leaves, a difference that was particularly pronounced compared with wound inoculation (see Fig. S3 in the supplemental material).

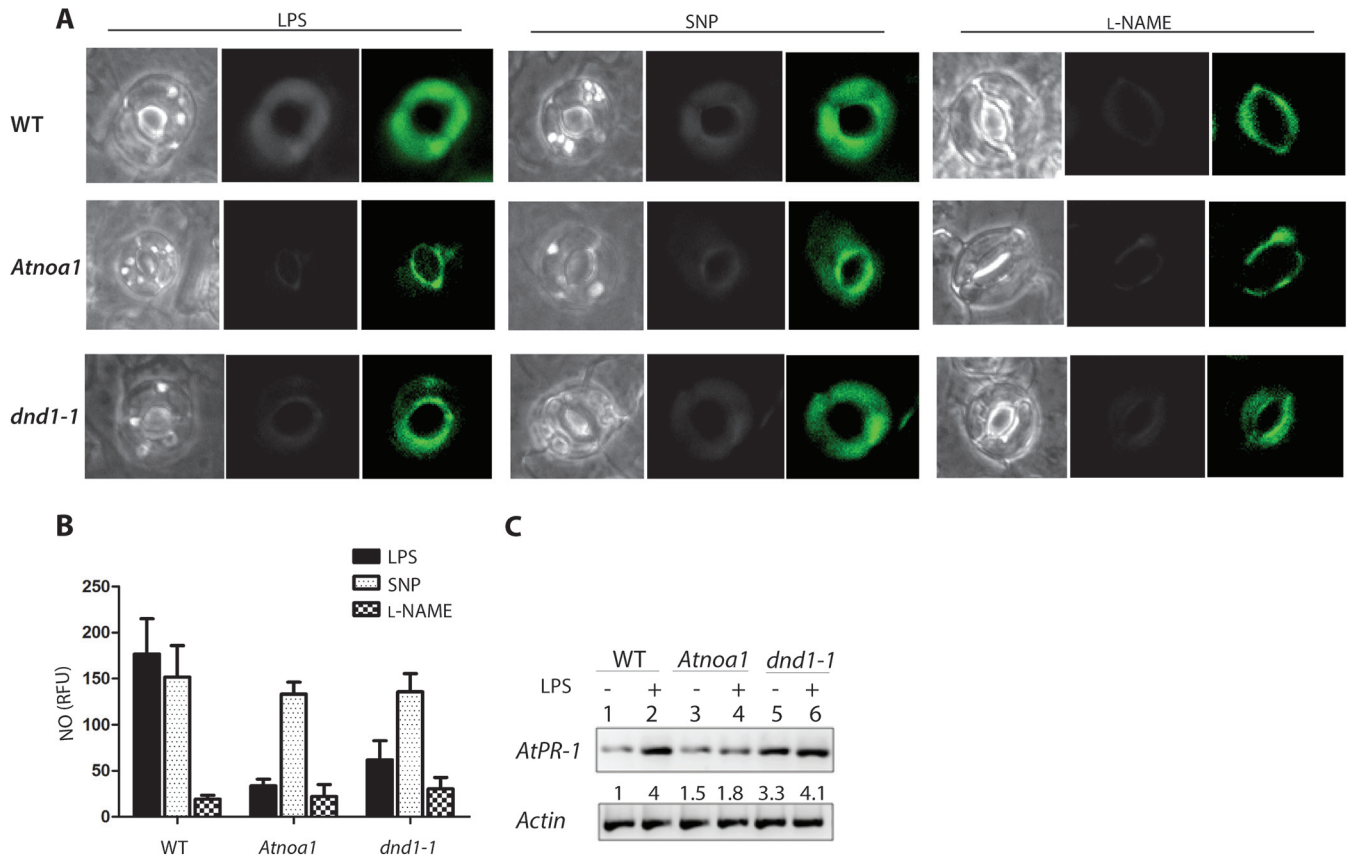
Since posttranslational modification of flagellin by glycosylation affects the virulence of *Pseudomonas syringae* in tobacco plants (39, 40), we compared the abilities of the parental and  $\Delta flmQ$  ( $\Delta bcal0111$ ) *B. cenocepacia* strains (Fig. 2A) to cause disease in *A. thaliana*. The *flmQ* gene encodes a predicted flagellin glycosyltransferase in *B. cenocepacia* (33). In comparison to the parental strain, the  $\Delta flmQ$  mutant was less pathogenic for *A. thaliana* (Fig. 2A) and exhibited reduced bacterial survival (Fig. 2B). Further, the  $\Delta flmQ$  mutant caused significantly less pathology on the leaves, with less than 30% of plants having disease symptoms, although slight chlorosis was observable (Fig. 2C), suggesting that nonglycosylated flagellin retains its elicitor activity. The reduced virulence of the  $\Delta flmQ$  mutant on *A. thaliana* leaves also correlated with bacterial defects in swarming and swimming (Fig. 3B and C), consistent with our previous report indicating that glycosylation affects bacterial motility (33). Together, these experiments demonstrate that both L-Ara4N modification of LPS and flagellin glycosylation are important for infectivity in *A. thaliana*.



**FIG 4** Role of L-Ara4N in NO generation in *Arabidopsis* guard cells. (A) Leaf epidermal peels prepared from parental (WT) plants were loaded with the buffer control (top panel) or with the NO-sensitive dye DAF-2DA (middle panel) prior to incubation with 100  $\mu\text{g}/\text{ml}$  ultrapure LPS from *E. coli* (bottom panel). (B) Leaf epidermal peels prepared from WT plants were treated with LPS isolated from the K56-2 or K56-2  $\Delta\text{arnT}$  strains. This experiment was repeated a total of two times. In each experiment, a minimum of three epidermal peels were used as treatment replicates. (C) Fluorometric quantification of NO generated in WT *Arabidopsis* leaves. (D) Induction of *PR-1* gene expression in *Arabidopsis* leaves by LPS. *Arabidopsis* leaves were treated with buffer (lane 1), K56-2 LPS (lane 2), or LPS isolated from the K56-2  $\Delta\text{arnT}$  strain (lane 3) for 24 h followed by RNA preparation. The actin gene was used as an internal control for RT-PCR. The numbers shown represent relative levels of *PR-1* being normalized relative to the control by densitometry.

**LPS induces robust and rapid nitric oxide burst in *A. thaliana*.** The reduced pathogenicity of the  $\Delta\text{arnT}$  *lptG*<sub>D31H</sub> mutant could be due to reduced bacterial survival during the initial stages of infection or, alternatively, poor recognition of LPS devoid of L-Ara4N by the plant innate immune system. We therefore investigated the levels of NO production in *A. thaliana* guard cells upon exposure to *B. cenocepacia* LPS from mutant and parental sources. Guard cells are specialized epidermal cells in the leaf epidermis surrounding stomatal pores that come into close contact with bacterial pathogens. LPS typically induces NO generation in *A. thaliana* (20, 34). An NO burst was detected *in vivo* using the NO-specific fluorescent dye diaminofluorescein diacetate (DAF-2DA). Treatment of guard cells with the ultrapure *Escherichia coli* LPS control resulted in a rapid burst of green fluorescence, indicative of NO production (Fig. 4A). Robust LPS induction of NO in intact guard cells could be reproduced using phenol-extracted LPS from both wild-type (WT) K56-2 and a mutant strain lacking L-Ara4N (Fig. 4B). Quantification

of the NO burst using a spectrofluorometric assay that detects NO accumulation indicated that LPS devoid of L-Ara4N has similar levels of elicitor activity, suggesting that LPS modification does not affect the innate immune response in *Arabidopsis* *in vivo*. Further, we compared LPS-induced NO production in guard cells isolated from parental *A. thaliana* and the *Atnoa1* and *dnd1* mutants. *Atnoa1* is a loss-of-function mutant in nitric oxide-associated protein 1 (NOA1), which is required for arginine-dependent NO generation and involved in signal cascades responding to PAMPs (20, 41). The *dnd1-1* (defense no death 1) mutant has a null mutation in the *CNGC2/DND1* gene and displays reduced NO generation (34, 42). Minimal responses were detected in *Atnoa1* and *dnd1* guard cells compared to wild-type cells (Fig. 5A, left panel). LPS-induced NO production was reduced dramatically in cells treated with the NOS inhibitor N<sup>G</sup>-nitro-L-Arg-methyl ester (L-NAME) (Fig. 5A, right panel). Similarly, LPS-dependent NO generation was not observed in *dnd1-1* or *Atnoa1* mutants after treatment with L-NAME, while incubation with



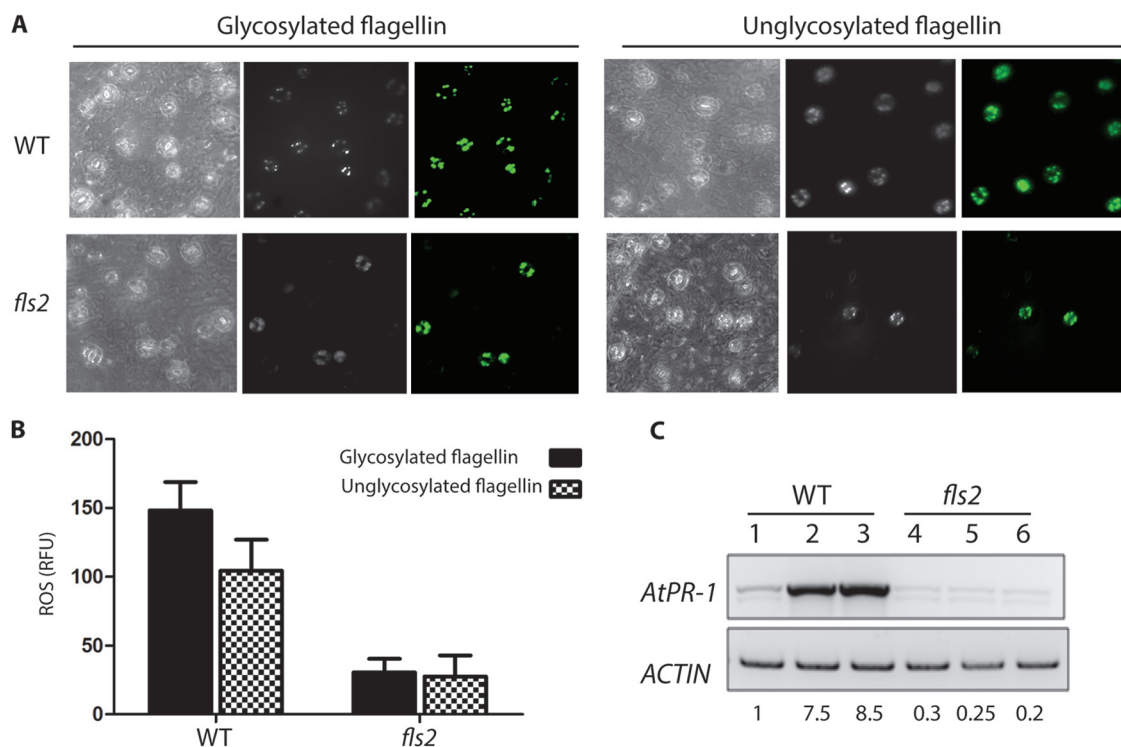
**FIG 5** LPS activates NO production in WT and *Atnoa1* and *dnd1-1* mutant guard cells. (A) Leaf epidermal peels prepared from WT (top panels) or *Atnoa1* (middle panels) or *dnd1-1* (bottom panels) mutant plants were loaded with 5  $\mu$ M DAF-2DA prior to incubation in reaction buffer alone (buffer control), 100  $\mu$ g/ml LPS (left panel), 50  $\mu$ M SNP (middle panel), or 200  $\mu$ M L-NAME (right panel). In each case, corresponding fluorescence and bright-field images are shown; the area of the peel subjected to analysis was greater than that shown in each case. This experiment was repeated three times using at least three epidermal peels per experiment. (B) Fluorometric quantification of NO generated in leaves of WT and *Atnoa1* and *dnd1-1* mutant *Arabidopsis* seedlings. (C) Induction of *PR-1* gene expression in *Arabidopsis* leaves by LPS. *Arabidopsis* leaves were treated with buffer (lanes 1, 3, and 5) or LPS (lanes 2, 4, and 6) for 24 h followed by RNA preparation. RT-PCR was performed with cDNAs for the *PR-1* and actin genes. The numbers between panels represent levels of *PR-1* being normalized relative to the control by densitometry.

the NO donor sodium nitroprusside (SNP) resulted in green fluorescence in the parental *A. thaliana* strain and both mutants. Therefore, the reduced NO burst in *Atnoa1* and *dnd1-1* cells was due to a defect in NO production (Fig. 5A, middle panel). NO was quantified in LPS-treated leaves of parental and mutant plants using a fluorometric assay. LPS treatment yielded 180 relative fluorescence units (RFU) in parental leaves, compared with 78% and 66% reductions in RFU observed for the *Atnoa1* and *dnd1-1* mutants, respectively (Fig. 5B). LPS-induced NO production in parental leaves was repressed by 6-fold upon treatment with the NO synthase inhibitor L-NAME (Fig. 5B). In contrast, parental and mutant leaves showed similar RFU in the presence of SNP. Together, these results demonstrate that *B. cenocepacia* LPS triggers a robust NO burst in *Arabidopsis* leaves, and both intact LPS and LPS lacking L-Ara4N can be equally recognized by the plant innate immune system.

**Flagellin perception in *A. thaliana* occurs irrespective of the flagellin glycosylation status.** We also investigated if the glycosylation status of the *B. cenocepacia* flagellin alters the immunity response of *A. thaliana*, which involves a rapid and transient burst of ROS that is subsequently associated with programmed cell death (43). Treatment of plants with *B. cenocepacia* flagellin triggered ROS generation in parental *A. thaliana* leaves but not in

leaves from the *FLS2* mutant, which lacks the flagellin receptor (Fig. 6A). This result agrees with previous reports in *Arabidopsis* and tomato plants demonstrating the importance of flagellin perception for ROS production and disease resistance (13, 15). Fluorometric quantification of ROS in parental and *fls2* mutant leaves showed a 3-fold reduction, indicating that flagellin recognition was necessary for eliciting ROS production (Fig. 6B). However, no significant differences were detected in ROS production by leaves treated with nonglycosylated flagellin, despite the fact that infection with bacteria lacking glycosylated flagellin produces fewer symptoms of infection (Fig. 2C). This result suggests that reduced pathogenicity of *A. thaliana* exposed to the *B. cenocepacia*  $\Delta$ *flmQ* mutant is due to reduced bacterial motility rather than differential perception of glycosylated versus nonglycosylated flagellin by the FLS2 receptor.

**Induction of pathogenesis-related gene 1 (*PR-1*) by LPS and flagellin.** Host defense responses in *A. thaliana* depend on ROS and NO production and induction of host defense or stress-associated genes, such as genes encoding pathogenesis-related proteins (20). We investigated *PR-1* gene expression by reverse transcription-PCR (RT-PCR), since the expression of this gene is considered a hallmark of defense response in plants (20, 21). *PR-1*

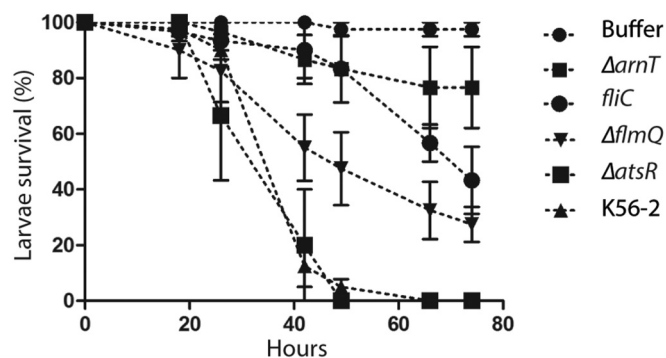


**FIG 6** Flagellin activates ROS generation in guard cells of WT and *fls2* mutant leaves. (A) Leaf epidermal peels prepared from WT (top panels) or *fls2* (bottom panels) plants were loaded with the ROS-sensitive dye DCF prior to incubation with glycosylated (left panel) or unglycosylated flagellin (right panel) from K56-2. In each case, corresponding fluorescence and bright-field images are shown; images are representative of three individual experiments. In each experiment, at least three epidermal peels were used as treatment replicates. (B) Fluorometric quantification of ROS generated in leaves of WT and *fls2* mutant *Arabidopsis* seedlings. (C) Induction of *PR-1* gene expression in *Arabidopsis* leaves by flagellin. *Arabidopsis* leaves were treated with buffer (lanes 1 and 4), WT (lanes 2 and 5), or unglycosylated flagellin (lanes 3 and 6) for 24 h followed by RNA preparation. RT-PCR was performed with cDNAs for the *PR-1* and actin genes. The numbers at the bottom represent the levels of *PR-1* being normalized relative to the control by densitometry.

expression was significantly increased in parental *A. thaliana* leaves 24 h after treatment with either intact LPS or LPS devoid of L-Ara4N LPS (Fig. 4D). In contrast, *PR-1* mRNA accumulation was reduced in *Atnoa1* mutant plants (Fig. 5C, lanes 3 and 4) suggesting a functional link between LPS-induced NO production and *PR-1* expression. Similarly, *PR-1* expression is activated by flagellin irrespective of its glycosylation status in parental *A. thaliana* but not in the flagellin-insensitive *fls2* mutant (Fig. 6C). As expected, *dnd1-1* mutant plants expressed high levels of *PR-1* mRNA, which is due to the constitutively active salicylic acid signaling pathway in this mutant (44, 45). Together, our findings indicate that modified LPS and flagellin induce *PR-1* transcription at levels similar to that of intact LPS and flagellin. This agrees with our results regarding induction of the oxidative burst, indicating that L-Ara4N modification of LPS and flagellin glycosylation are critical for infectivity in *A. thaliana* but do not alter the perception of these molecules by the plant innate immune receptors.

**Comparison of *Arabidopsis* and *Galleria* infection models.** The previous results indicate that flagellin and LPS modifications alter the infectivity of *B. cenocepacia* in *A. thaliana*, but the plant can perceive these molecules irrespectively. Therefore, we tested the ability of *B. cenocepacia* strains utilized in the plant infection model for survival and pathogenicity in *G. mellonella*. The *G. mellonella* moth larvae have been increasingly used as an infection model for many bacterial pathogens, including several *Burkholderia* species (8, 46, 47). *B. cenocepacia* parental and mutant strains

were injected into *G. mellonella*, and survival was monitored for over 72 h. As with *A. thaliana*, K56-2  $\Delta$ *atsR* exhibited high virulence denoted by no larvae surviving at 48 h postinjection with  $10^4$  CFU (Fig. 7), compared to the  $5\% \pm 3\%$  survival of larvae infected with the parental K56-2 strain. In contrast, the  $\Delta$ *arnT* *lptG*<sub>D31H</sub> strain was greatly attenuated, as shown by larval survival rates of more than 80% at 48 h postinfection (Fig. 7). At infection



**FIG 7** Virulence of *B. cenocepacia* strains in *G. mellonella*. Larvae were infected with  $1 \times 10^4$  CFU of the indicated *Burkholderia* strains. Larval survival was monitored during 75 h postinfection. The data are means  $\pm$  standard errors of the means (SEM). All bacteria grew at similar rates in Luria-Bertani medium (see Fig. S5 in the supplemental material).



doses of  $10^4$  CFU, similar attenuation levels were observed in the *fliC* mutant, while the  $\Delta flmQ$  mutant showed an intermediate phenotype ( $50\% \pm 13.5\%$  survival). At 72 h postinfection, the larval survival rates with the  $\Delta arnT$  *lptG*<sub>D31H</sub>, *fliC*, and  $\Delta flmQ$  strains were  $77\% \pm 14\%$ ,  $43\% \pm 12\%$ , and  $28\% \pm 7\%$ , respectively. Control larvae injected with buffer remained viable until the end of the experiment. These results are comparable to the data obtained from the *A. thaliana* infections, confirming the significance of flagellin glycosylation and L-Ara4N modification of LPS in the infectivity of *B. cenocepacia*.

## DISCUSSION

Virulence and pathogenicity require considering both the host and the microbe; the use of these terms to define a microbe in isolation of its host has recently been debated (48, 49). Opportunistic bacteria are a classic example of microbes whose ability to cause disease is intimately related to the host's ability to recognize and respond to the infection. However, the microbe's capacity to establish a niche upon entering the host is a critical feature associated with infection. We show here that modifications in the LPS molecule and flagellin glycosylation do not affect recognition by plant innate immune receptors but are still required for bacteria to establish an infection. Our experiments using the *B. cenocepacia* K56-2-*A. thaliana* infection model reveal that plant seedlings sense LPS and flagellin as PAMPs and induce a burst of NO and ROS that contributes to basal resistance and eventually leads to a hypersensitive response against *B. cenocepacia*. Redox signaling has emerged as a main regulator of cellular function in plant pathophysiology (50). Pathogen recognition turns on a signaling cascade in which free radicals, such as NO and ROS, act as "infochemicals" for activation of various defense genes that mediate disease resistance in plants (14). Production of NO and ROS upon K56-2 LPS or flagellin treatment potentiated the expression of *PR-1*, which is a disease resistance marker. In contrast, *PR-1* gene expression was reduced when *Atnoa1* and *fls2* mutant plants were treated with LPS or flagellin, indicating that LPS-responsive genes are NO inducible (20). In our work, we did not observe a distinguishable difference in the levels of expression of the *PR-1* gene after treatment of leaves with intact or modified versions of LPS and flagellin.

Although we did not use purified lipid A in our study, these observations contrast with a previous report indicating that isolated lipid A of a nonpathogenic mutant of *Xanthomonas campestris*, consisting mainly of penta-acylated lipid A with phosphoethanolamine substitutions of its phosphate groups, was unable to elicit *PR-1* expression (23). The authors concluded that the substitution of both lipid A phosphate groups with phosphoethanolamine would neutralize the net negative charge of the lipid A, which could affect binding to putative plant receptors (23). In contrast, the *B. cenocepacia* lipid A phosphate groups are substituted by L-Ara4N molecules, and disappearance of these residues in the  $\Delta arnT$  *lptG*<sub>D31H</sub> mutant would expose negative charges, thus maintaining the ability of lipid A to activate *PR-1* expression. Importantly, the lipid A of *B. cenocepacia* is tetra- and penta-acylated, but the acyl chains are longer than those of the *X. campestris* lipid A (51). Therefore, the fact that parental LPS and LPS devoid of L-Ara4N from *B. cenocepacia* induce similar levels of *PR-1* gene expression suggests that the acyl chain composition may also play a role in LPS detection by plant innate immune receptors.

Irrespective of K56-2 LPS elicitor activity, *B. cenocepacia* mu-

tants unable to produce L-Ara4N-modified LPS have extremely high sensitivity to polymyxin B (27), suggesting that they would also be highly sensitive to plant antimicrobial peptides. Further, we show that posttranslational modification of flagellin by glycosylation affects bacterial motility and virulence in *Arabidopsis* but does not significantly alter the elicitor activity of the protein. A correlation between defects in flagellin glycosylation in *Pseudomonas syringae* and reduced ability to induce HR in plants has been reported (39, 40), but the molecular mechanism is unknown. Our experiments indicate that reduced virulence but increased activation of plant defenses by the  $\Delta flmQ$  mutant producing nonglycosylated flagellin is due to bacterial motility defects that prevent bacteria to establish infection. This could be clearly demonstrated by showing that the attenuation of the K56-2 *fliC* mutant strain is bypassed if bacteria are directly infiltrated into the plant's deeper tissues. A role for flagellar motility was also reported for several plant pathogens, such as *Erwinia carotovora* (52), *Ralstonia solanacearum* (53), and several pathovars of *P. syringae* and *Xanthomonas campestris* (54–56).

In conclusion, we demonstrate that flagellin glycosylation and LPS modification with L-Ara4N play a significant role in bacterial survival during the early stages upon infection but do not alter the perception of these molecules by the plant innate immune receptors, indicating these modifications are only critical to establish infection. Our experiments therefore illustrate the notion that the microbe's perception by the host and establishment of infection are interrelated but independent events.

## MATERIALS AND METHODS

**Plants and growth conditions.** Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) and the *Atnoa1* (SALK no. CS6511), *fls2* (SALK no. 121477), and *dnd1-1* (SALK no. 066908C) mutants were surface sterilized by being sequentially soaked in 75% ethanol for 1 min, rinsed three times with sterile water, soaked in 20% bleach for 15 min, and rinsed five times with sterile water. Sterilized seeds were kept at 4°C in the dark for 3 days for vernalization and were planted in magenta boxes containing Murashige and Skoog basal salt mixture (Sigma-Aldrich) supplemented with 1% sucrose (wt/vol) and 0.8% agar, and placed in a growth chamber at 22°C with a light intensity of  $80 \mu\text{E m}^{-2} \text{s}^{-1}$ . Seedlings were grown under 16 h of light and 8 h of darkness for 21 days. Mutants were obtained from the *Arabidopsis* Biological Resource Center (ABRC); tobacco plants (*Nicotiana benthamiana*) were grown at 26°C with a 16-h photoperiod.

**Bacterial strains and growth conditions.** *B. cenocepacia* K56-2 and its isogenic mutants (Table 1) were cultured at 37°C in Luria-Bertani (LB) broth overnight. *B. cenocepacia* K56-2 is a clinical isolate that belongs to the ET-12 epidemic strain lineage (57). Strain MSS25 carries a polar insertion in the *fliC* gene, which prevents the synthesis of the flagellin filament and also likely inactivates the downstream genes *fliD1* and *fliT*, encoding a flagellar hook and an accessory protein, respectively (35). To analyze the growth rate of mutants, overnight cultures were diluted into fresh medium at a starting optical density at 600 nm (OD<sub>600</sub>) of 0.05. Samples were then aliquoted in a 100-well honeycomb microtiter plate, and growth rates were followed by measuring the OD<sub>600</sub> every hour under continuous shaking for 24 h in a Bioscreen C automated growth curve analyzer (MTX Lab Systems, Vienna, VA).

**Plant inoculation methods.** Five- to 6-week-old *A. thaliana* or *Nicotiana benthamiana* plants were used for the initial inoculation experiments. For soil drainage infection, plants were watered with bacterial suspensions in MES (4-morpholineethanesulfonic acid sodium salt) buffer. For the surface spraying method, bacteria from overnight cultures were suspended in 10 mM MES buffer at different CFU. For inoculation by the syringe-infiltration method, bacteria were infiltrated into the leaves using a needleless syringe. For wound inoculation, seedlings were wounded at

the leaf surface by being scratched with a 20-gauge needle. Wounded seedlings were immediately inoculated with bacterial suspensions. MS agar plates containing inoculated seedlings were sealed with Parafilm and placed in growth chambers for 7 days. Plants were monitored for disease symptoms daily. At day 7, three leaves from each plantlet were excised, washed with 5% bleach for 1 min, and rinsed with sterile water. The leaf was blotted dry on sterile filter paper and imprinted on LB agar plates to determine if there were any bacteria on the surface of the leaves. The imprinted plates were incubated at 37°C for 24 h. The leaves were then weighed and macerated in 500  $\mu$ l phosphate-buffered saline (PBS) with a micropestle, serially diluted, and plated on LB agar plates in triplicates. Only leaf samples that did not show any bacterial growth on the imprinted plates were counted to avoid counting contaminating bacteria from leaf surfaces. Other mutants were similarly infiltrated into leaves, and HR symptoms were visualized by bleaching the leaves in ethanol (Fig. 2C). Ethanol bleaching was used to provide enhanced visualization of HR development in leaves and facilitate comparison with the parental strain.

**RNA isolation, cDNA synthesis, and RT-PCR.** Total RNA was extracted from frozen leaves of *A. thaliana* using the SV total RNA isolation system (Promega), and 1  $\mu$ g of RNA was reverse transcribed using the QuantiTect reverse transcription kit (Qiagen). The coding sequences of the *PR-1* and actin genes, available through GenBank, were used to design the following primers: forward, 5'-GATGTGCCAAAGTGAGGTG-3', and reverse, 5'-CTGATACATATACACGTCC-3', to amplify *PR-1* and forward, 5'-TGCTCTTCTCATGCTAT-3', and reverse, 5'-ATCCTCCGATCCAGACACTG-3', to amplify the actin gene. PCR was carried out with an initial denaturation step at 94°C for 4 min, followed by 30 cycles of denaturation (30 s at 94°C) for *PR-1* and 21 cycles for the actin gene, annealing (30 s at 53°C), and extension (1 min at 72°C). After the last cycle, a final extension was carried out for 5 min at 72°C. PCR products were visualized on 1% agarose gels using UV light in a Gel-Doc system (Alpha Innotech). mRNA levels of *PR-1* were normalized relative to the control and internal control by densitometry using ImageJ.

**LPS extraction, MS, and flagellin purification.** LPS was extracted from equal biomass using the proteinase K method as previously described (58). Extracted LPS was dissolved at 1 mg/ml in water containing 2.5 mM MgCl<sub>2</sub> plus 1 mM CaCl<sub>2</sub> and shaken for 3 h on a rotary shaker. If not mentioned otherwise, experiments were performed with K56-2 LPS or with buffer A containing 1.0 mM CaCl<sub>2</sub> and 2.5 mM MgCl<sub>2</sub> (pH 7.6) as a control. Flagellin purification was performed as described earlier (33). Lipid A of strains MH55 and K56-2 (Table 1) was examined by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Cells were grown overnight in Luria-Bertani broth, centrifuged (10,000  $\times$  g), washed twice with phosphate buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.7 mM KH<sub>2</sub>PO<sub>4</sub>), and freeze-dried. Lipid A was extracted and desalted, as previously described (59). For MS analysis, dihydroxybenzoic acid (DHB) (Sigma Chemical Co., St. Louis, MO) was used as a matrix and prepared to saturation in acetonitrile–0.1% trifluoroacetic acid (1:2 vol/vol). A 2- $\mu$ l sample was loaded on the target, dried, covered by 1  $\mu$ l of DHB, and inserted in a Bruker Autoflex MALDI-TOF spectrometer. Data were acquired and analyzed using the Flex Analysis software.

**In vivo NO and ROS analysis.** NO was measured in guard cells of leaf epidermal peels prepared from leaves of 3-week-old plants (grown as described above) using diamino fluorescein diacetate (DAF-2DA) fluorescence as described by Guo et al. (60), with the following modifications. The epidermal peels were submerged in buffer (pH 5.7) containing 5 mM MES-KOH, 0.25 mM KCl, and 1 mM CaCl<sub>2</sub> with 5  $\mu$ M DAF-2DA and transferred either to reaction buffer containing 100  $\mu$ g/ml LPS or preincubated for 10 min in reaction buffer containing 50  $\mu$ M sodium nitroprusside (SNP) or 200  $\mu$ M N<sup>G</sup>-nitro-L-Arg-methyl ester (L-NAME) and then for 20 min with LPS. The epidermal peels were placed underneath a coverslip on a microscope slide with several drops of reaction mixture. In a similar way, ROS was detected by 2  $\mu$ M 2,7-dichlorofluorescein diacetate (DCF) and flagellin treatment following the incubation of leaf epidermal leaves in MES-KOH buffer for 2 h.

**Microscopy.** Fluorescence and phase-contrast images were acquired using a QImaging Retiga-SRV camera on an AxioScope 2 (Carl Zeiss).

**Fluorometric quantification of NO and ROS.** To monitor NO accumulation in *Arabidopsis* leaf peels, the DAF-2DA fluorescence was measured at excitation of 480 nm and emission of 521 nm, using a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Mississauga, Ontario, Canada). The plate was rocked for 20 s before measuring. Similarly, to determine accumulation of ROS, DCF was added to a final concentration of 2  $\mu$ M. Fluorescence was measured at excitation of 480 nm and emission of 521 nm in 96-well white plates. The background fluorescence of each probe in the buffer control was subtracted. Autofluorescence of the buffer, without adding the probes, was measured and corrected by subtraction from the fluorescence signals. The suspensions were protected from light throughout the assays to avoid photo-oxidation.

**Bacterial swarming and swimming motility assays.** Swarming motility assays were performed as described previously (37). Assays were done in triplicate and repeated independently three times. Bacterial swimming motility was analyzed on soft agar plates (1% Bacto tryptone in 0.3% agar). The OD<sub>600</sub> of overnight cultures was adjusted to 1, and 2  $\mu$ l of culture was inoculated in the center of the agar plate. The diameter of the growth zone was measured after 24 h of incubation at 37°C.

**Galleria mellonella infection model.** *G. mellonella* larvae were obtained from Recorp and stored in the dark at 4°C. Injections of 10  $\mu$ l containing 1  $\times$  10<sup>4</sup> CFU diluted in 10 mM MgSO<sub>4</sub>·7H<sub>2</sub>O supplemented with 100  $\mu$ g ampicillin  $\mu$ l<sup>-1</sup> were given to the larvae into the hemocoel through the hindmost right proleg. Infected larvae were incubated at 30°C, and larval survival was monitored up to 75 h as judged based lack of movement in response to stimuli. Control larvae were injected with 10  $\mu$ l of the same buffer without bacteria. Ten larvae were used for each condition, and the experiment was repeated on three independent occasions.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00679-15/-/DCSupplemental>.

- Figure S1, PDF file, 0.4 MB.
- Figure S2, PDF file, 0.1 MB.
- Figure S3, PDF file, 0.1 MB.
- Figure S4, PDF file, 0.4 MB.
- Figure S5, PDF file, 0.1 MB.

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