Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants

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**SUMMARY**

*Pseudomonas putida* WCS358 is a plant growth-promoting rhizobacterium originally isolated from the rhizosphere of potato. It can suppress soil-borne plant diseases by siderophore-mediated competition for iron, but it has also been reported to result in induced systemic resistance (ISR) in *Arabidopsis thaliana*. Bacterial determinants of this strain involved in inducing systemic resistance in *Arabidopsis* were investigated using a Tn5 transposon mutant defective in biosynthesis of the fluorescent siderophore pseudobactin, a non-motile Tn5 mutant lacking flagella, and a spontaneous phase-resistant mutant lacking the O-antigenic side chain of the lipopolysaccharides (LPS). When using *Pseudomonas syringae pv. tomato* as the challenging pathogen, purified pseudobactin, flagella and LPS all triggered ISR. However, the mutants were all as effective as the parental strain, suggesting redundancy in ISR-triggering traits in WCS358. The *Botrytis cinerea*–*tomato*, *B. cinerea*–*bean* and *Colletotrichum lindemuthianum*–*bean* model systems were used to test further the potential of *P. putida* WCS358 to induce ISR. Strain WCS358 significantly reduced disease development in all three systems, indicating that also on *tomato* and *bean* WCS358 can trigger ISR. In both *tomato* and *bean*, the LPS mutant had lost the ability to induce resistance, whereas the flagella mutant was still effective. In *bean*, the pseudobactin mutant was still effective, whereas this mutant has lost its effectiveness in *tomato*. In both *bean* and *tomato*, flagella isolated from the parental strain were not effective, whereas LPS or pseudobactin did induce systemic resistance.

**INTRODUCTION**

All plants possess active defence mechanisms against pathogen attack. If defence mechanisms are triggered by a stimulus prior to infection by a virulent plant pathogen, disease can be reduced (for review see van Loon et al., 1998). Induced disease resistance can be defined as the process of active resistance dependent on the host plant’s physical or chemical barriers, activated by biotic or abiotic agents (Kloepper et al., 1992). The resulting elevated state of resistance in plant parts distant from the site of primary triggering is variably referred to as systemic acquired resistance (SAR) or induced systemic resistance (ISR). The term SAR is commonly used to denote systemic resistance induced by pathogens. SAR is characterized by an early increase in endogenously synthesized salicylic acid (SA), which appears to be an essential signalling molecule in the SAR pathway (Métraux et al., 1990). Accumulation of SA appears to be critical for the induction of the SAR-signalling pathway because transgenic plants unable to accumulate SA are incapable of developing SAR (Gaffney et al., 1993). Selected non-pathogenic plant growth-promoting rhizobacteria (PGPR) are also known to induce a systemic resistance (Bakker et al., 2003; Kloepper et al., 1992; van Loon et al., 1998; Van Peer et al., 1991; Wei et al., 1991). To differentiate this type of induced resistance from pathogen-induced SAR, the term rhizobacteria-mediated ISR is used.

Bacterial determinants of ISR that have been identified are lipopolysaccharides (LPS), iron-regulated compounds, and certain antibiotics. LPS of *Pseudomonas fluorescens* strain WCS417 has been implicated in triggering ISR against *Pseudomonas syringae pv. tomato* in *Arabidopsis* (Van Wees et al., 1997), and to Fusarium wilt in carnation (Van Peer and Schippers, 1992), radish (Leeman et al., 1995) and tomato (Duijff et al., 1997). In radish, the O-antigenic side chains of the LPS of both *P. fluorescens* WCS417 and *P. fluorescens* WCS374 were major determinants of ISR against Fusarium wilt under iron-replete conditions, but not under iron-limiting conditions. Unknown iron-regulated factors in WCS374 and WCS417 caused ISR under low-iron conditions. Likewise, in *Pseudomonas putida* BTP1, an unknown iron-regulated metabolite Cx appeared to be responsible for ISR in *bean* against *Botrytis cinerea* (Ongena et al., 2002).

De Meyer and Höfte (1997) found that the SA-producing PGPR *Pseudomonas aeruginosa* 7NSK2 triggered ISR against *B. cinerea*
in bean when the bacterium was cultivated on iron-poor media but not on iron-rich media, and that SA-negative mutants of this strain had lost their ability to induce systemic resistance. SA production by *P. aeruginosa* 7NSK2 appeared to be essential for ISR also to tobacco mosaic virus (TMV) in tobacco (De Meyer et al., 1999) and to *Colletotrichum lindemuthianum* in bean (Bigirimana and Höfte, 2002). Likewise, expression of SA-biosynthetic genes in *Pseudomonas fluorescens* P3 improved induction of resistance to tobacco necrosis virus (TNV) in tobacco (Maurhofer et al., 1998). However, in *Pseudomonas fluorescens* CHA0 the siderophore pyoverdine, rather than SA, appeared to be implicated in induced resistance against TNV (Maurhofer et al., 1994). Similarly, in the SA-producing PGPR *Serratia marcescens* 90-166, an unidentified catechol-type siderophore rather than SA is involved in ISR (Press et al., 1997, 2001).

Antibiotics can also be involved in ISR. In *Arabidopsis*, Iavicoli et al. (2003) recently demonstrated the importance of 2,4-diacetylphloroglucinol (DAPG) produced by *P. fluorescens* CHA0 for the induction of resistance against *Peronospora parasitica*. In tomato, Audenaert et al. (2002b) showed that the phenazine antibiotic pyocyanin in combination with SA or the SA-containing siderophore pyochelin produced by *P. aeruginosa* 7NSK2 act as determinants for induced resistance against *B. cinerea*.

*Pseudomonas putida* WCS358 is a PGPR originally isolated from the rhizosphere of potato (Geels and Schippers, 1983a, b). It can suppress soil-borne plant diseases by siderophore-mediated competition for iron (Bakker et al., 1986, 1993). WCS358 can also induce systemic resistance in *Arabidopsis thaliana* against *Pseudomonas syringae pv. tomato* and *Fusarium oxysporum* f. sp. *raphani* (Van Wees et al., 1997), but the strain is unable to induce resistance against Fusarium wilt in carnation (Duijff et al., 1993) or radish (Leeman et al., 1995).

In the present study, Arabidopsis, bean and tomato model systems were compared to explore further the potential of *P. putida* WCS358 to induce ISR and to identify bacterial determinants involved in this process.

**RESULTS**

The ability of *P. putida* WCS358 to trigger ISR in *A. thaliana*, bean and tomato was compared. To study bacterial determinants involved in induced systemic resistance two strategies were used. The first involved testing specific knock-out mutants of WCS358 for their ability to induce resistance relative to the parental strain. Conversely, the corresponding bacterial components, pseudobactin, LPS-containing cell-wall preparations and flagella, were applied to the plants roots, and leaves were challenged with the pathogens.

**Resistance induced in Arabidopsis**

In Arabidopsis, WCS358 reduced symptoms of *P. syringae pv. tomato* by 20–30% (Fig. 1). Purified pseudobactin was as effective in inducing resistance as live bacteria. However, the mutant defective in pseudobactin biosynthesis (PB-) was equally effective in suppressing the disease. Likewise, the bacterial cell-wall preparations suggested a role of LPS, but the mutant lacking the O-antigen side chain (OA-) still induced systemic resistance, and the flagella mutant (Fla-) and isolated flagella of WCS358 were as effective as the parental strain (Fig. 1).

**Effects on the severity of anthracnose and grey mould**

Treatment of bean roots with wild-type strain WCS358 significantly reduced disease development resulting from *C. lindemuthianum*. Both the flagella mutant (Fla-) and the siderophore mutant (PB-) induced systemic resistance, whereas the LPS mutant (OA-) was not effective (Fig. 2A). Comparable results were obtained when WCS358 and its mutants were applied to bean roots and the leaves were challenged with *B. cinerea* (Fig. 3A). When isolated compounds of WCS358 were tested, flagella were not effective in inducing resistance, whereas both LPS containing cell walls and pseudobactin induced systemic resistance to *C. lindemuthianum* (Fig. 2B) and *B. cinerea* (Figs 3B and 4B).

In tomato the number of *B. cinerea* spreading lesions was significantly reduced on leaves from the plants whose roots were treated with WCS358 or the flagella mutant Fla-. Root inoculation with PB- or OA- had no significant effect on the *B. cinerea* infection (Figs 4C and 5A). However, when the pseudobactin and LPS mutant were combined, they were as effective as the parental strain in inducing resistance (Fig. 5B). The isolated LPS and pseudobactin induced resistance to *B. cinerea* in tomato, but flagella were not effective (Fig. 5C).
Bean and tomato root colonization by WCS358 and its mutants

To exclude the possibility that the inability of the LPS and/or pseudobactin mutant to induce systemic resistance is due to insufficient colonization of the rhizosphere, population densities of WCS358 and its mutants on bean and tomato roots were examined. All bacterial strains tested colonized bean and tomato roots to similar population densities (3.2–28 \times 10^5 c.f.u./g root fresh weight) (Table 1). The pseudobactin mutant (PB-) was present at even higher levels in the rhizosphere of both bean and tomato compared with the parental strain and the two other mutants. Moreover, the population densities are well above 10^5 c.f.u./g root, the threshold value of \textit{P. fluorescens} WCS374 for induction of systemic resistance in radish (Raaijmakers et al., 1995).

\section*{DISCUSSION}

\textit{Pseudomonas putida} strain WCS358 was able to induce systemic resistance in Arabidopsis against \textit{P. syringae pv. tomato}, in bean against \textit{Colletotrichum lindemuthianum} and \textit{B. cinerea} and in tomato against \textit{Botrytis cinerea}. Multiple determinants of \textit{P. putida} WCS358 were able to induce resistance in \textit{Arabidopsis thaliana}. Although application of pseudobactin, LPS-containing cell walls or flagella induced resistance, the mutants lacking pseudobactin, flagella or the O-antigenic side chain of LPS still induced resistance, indicating that if one trait is lacking in the bacterium, resistance is still induced through the other trait(s). In bean and tomato, pseudobactin- and LPS-containing cell walls induced resistance when applied to the plant roots. However, flagella of WCS358 were not effective in inducing resistance and the resistance-inducing capacities of the flagella mutant did not differ from the wild-type.

Bacterial flagellins, the principal structural component of flagella, can act as elicitors of defence responses in Arabidopsis.
Recently, the FLS2 receptor involved in flagella perception was identified in Arabidopsis (Felix et al., 1999; Gomez-Gomez and Boller, 2002; Gomez-Gomez et al., 2001; Montesano et al., 2003). FLS2 encodes a receptor-like kinase that is similar to TLR5, the Toll-like receptor involved in flagellin recognition in mammalian cells. FLS2 consists of a potential signal peptide, an extracellular LRR (leucine-rich repeats) domain, a transmembrane domain and a serine–threonine kinase domain (Gomez-Gomez and Boller, 2000). The flagellin interacts with the extracellular FLS2 LRR domain; this interaction leads to activation of the FLS2 kinase domain, which causes a rapid phosphorylation of many proteins ultimately leading to induced expression of defence genes (Asai et al., 2002). The elicitor-active epitope was localized to the most conserved domain in the N-terminal part of flagellin, and synthetic peptides spanning 15–22 amino acid residues of this domain (flg15, flg22) showed full biological activity as elicitors (Felix et al., 1999). It is likely that flagellin from WCS358 flagella is recognized by the FLS2 receptor in Arabidopsis, because the amino acid sequence of the conserved part of the N-terminal domain of P. putida flagellin is identical to flg15. Moreover, it was shown that FLS2 is expressed in roots of Arabidopsis plants (Gomez-Gomez and Boller, 2000). In addition, the rapid protein phosphorylation in response to flg22 appears to be independent of SA signalling. It has been shown that WCS358 induces resistance
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in Arabidopsis by an SA-independent pathway (Van Wees et al., 1997). Interestingly, the major extracellular protein of WCS358 corresponds to flagellin (de Groot et al., 1996). Apparently, the flagella are easily sheared from the cells and appear in the extracellular fraction. Alternatively, a substantial amount of flagellin may not be assembled in flagella but rather secreted (de Groot et al., 1996). Felix et al. (1999) state that intact flagella attached to bacteria are not expected to act as direct stimuli for perception by plants. Depolymerization or fragmentation of the flagellum is probably required to obtain elicitor-active flagellin subunits that can diffuse through the cell wall and reach the receptor on the plasma membrane. In bean and tomato inoculated with B. cinerea and/or C. lindemuthianum, flagella do not seem to be involved in ISR (Figs 2B and 5C). If we hypothesize that flagellin from WCS358 is recognized by the FLS2 receptor in Arabidopsis, it is presently unclear why purified flagella of WCS358 failed to induce resistance in tomato, as a high-affinity binding site for flagellin, flg22 and flg15, has been identified also in tomato (Felix et al., 1999; Meindl et al., 2000). It is possible that the resistance pathway induced upon flagellin-receptor recognition in tomato is not effective against B. cinerea. Nothing is known about the possible presence of a flagellin receptor in bean. In this context it is interesting to note that SA-negative mutants of Pseudomonas aeruginosa 7NSK2 that failed to induce systemic resistance in bean, tobacco and tomato (Audenaert et al., 2002b; De Meyer and Höfte, 1997; De Meyer et al., 1999) still induced systemic resistance to P. syringae pv. tomato in Arabidopsis by an SA-independent pathway (Ran, 2002). We are currently investigating whether flagellin from P. aeruginosa 7NSK2 is involved in this elicitation.

Table 1 Bean and tomato root colonization by WCS358 and its mutants lacking flagella (Fla-), pseudobactin (PB-) or the O-antigen of the LPS (OA-).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>c.f.u./g of root fresh weight (x10^5)</th>
<th>Stat. anal.* Bean</th>
<th>Stat. anal.* Tomato</th>
</tr>
</thead>
<tbody>
<tr>
<td>WCS358</td>
<td>18.0 ± 8.4 ab</td>
<td>7.5 ± 9.5 a</td>
<td></td>
</tr>
<tr>
<td>Fla-</td>
<td>21.7 ± 3.8 b</td>
<td>3.2 ± 6.6 a</td>
<td></td>
</tr>
<tr>
<td>PB-</td>
<td>28.0 ± 6.2 b</td>
<td>25.5 ± 3.4 b</td>
<td></td>
</tr>
<tr>
<td>OA-</td>
<td>10.2 ± 1.8 a</td>
<td>6.0 ± 2.0 a</td>
<td></td>
</tr>
</tbody>
</table>

*Treatments followed by a different letter are significantly different according to Kruskal–Wallis followed by Mann–Whitney (P = 0.05).

Fig. 5 (A) Influence of soil treatment with P. putida WCS358 and its mutants lacking flagella (Fla-), pseudobactin (PB-) or the O-antigen of the LPS (OA-) on the number of spreading lesions on tomato 5 days after inoculation with B. cinerea. Bars with a common letter do not differ significantly using logistic regression test at P = 0.05. (B) Influence of soil treatment with P. putida WCS358 and its mutants lacking pseudobactin (PB-) or the O-antigen of the LPS (OA-) (alone or in combination) on the number of spreading lesions on tomato 5 days after inoculation with B. cinerea. Bars with a common letter do not differ significantly using logistic regression test at P = 0.05. (C) Influence of soil treatment with P. putida WCS358, purified pseudobactin (PB), flagella (Fla) or LPS (OA) on the number of spreading lesions on tomato 5 days after inoculation with B. cinerea. Bars with different letters are significantly different using logistic regression test at P = 0.05.
that consists of repeated oligosaccharides. The Toll-like receptor TLR4 that recognizes the lipid A domain of Gram-negative bacterial LPS in mammalian systems is well characterized (for reviews see Raetz and Whitfield, 2002; Underhill and Ozinsky, 2002). However, the O-antigen, rather than the lipid A, seems to be involved in inducing systemic resistance in plants. Elicitor activity of the O-antigenic side chain of LPS was identified for Pseudomonas fluorescens WCS417 on Arabidopsis (Van Wees et al., 1997), carnation (Van Peer and Schippers, 1992), radish (Leeman et al., 1995) and tomato (Dujiff et al., 1997), as well as for WCS374 but not WCS358 on radish. Strains WCS358 and WCS374 differ in their LPS patterns (De Weger et al., 1987). Nothing is known about the mechanisms by which plants perceive LPS and how LPS activates plant defence responses (Newman et al., 2001). Neither is it known in what form the plant cells recognize LPS. Intact LPS may be perceived as an integral part of the bacterial surface or released in micelles or blebs (Beveridge, 1999). Alternatively, it is conceivable that portions of LPS are released from bacteria following degradation by bacterial or plant enzymes (Newman et al., 2001). It is likely that the O-antigen polysaccharides are recognized by receptors on the plasma membrane of plant roots and that this interaction triggers ISR.

Like LPS, purified WCS358 pseudobactin induced resistance in Arabidopsis, bean, and tomato. Leeman et al. (1995) showed that the pseudobactin of WCS358 did not induce resistance to Fusarium wilt of radish. In bean plants, the pseudobactin mutant PB- and purified pseudobactin of WCS358 were as effective as, or even more effective than, the wild-type. Likewise, the purified pseudobactin siderophore of WCS374 induced resistance in the Fusarium wilt–radish system, while a pseudobactin mutant of WCS374 still induced resistance under iron-replete and iron-limited conditions (Leeman et al., 1996). In tomato plants, however, the pseudobactin mutant PB- lost its ability to induce resistance whereas it significantly protected leaves against B. cinerea in combination with mutant OA- lacking the O-antigen of LPS. This means either that both LPS and pseudobactin are needed for ISR in tomato, or that their effect is additive. It is possible that the mutants produce too little pseudobactin or LPS to be effective in inducing resistance in the absence of the other compound. At present it remains unclear how pseudobactin triggers ISR.

Induction of systemic resistance by P. putida WCS358 is complex. In Arabidopsis it involves at least flagella, pseudobactin and LPS. The effect of these compounds seems to be complementary rather than additive, because all mutants induced resistance to the same level as the wild-type strain. This situation is reminiscent of innate immune responses in animals, where immune cells use many different Toll-like receptors to detect several determinants of a microorganism simultaneously. These so-called pathogen-associated molecular patterns (PAMPs), which trigger innate immune responses in vertebrate and invertebrate organisms, include the LPS of Gram-negative bacteria, bacterial flagellin, peptidoglycans from Gram-positive bacteria and fungal cell-wall-derived glucans, chitins, mannans and proteins. Interestingly, many of these molecules have long been known to act as general elicitors of defence responses in a multitude of plant species, indicating that these general elicitors are conceptually similar to PAMPs (Jones and Takemoto, 2004). These findings suggest that plants have acquired and maintained the ability to recognize microbe-associated patterns (Nürnberger et al., 2004). We have shown here that activation of ISR by WCS358 is due to recognition of ‘microbe-associated molecular patterns’ such as LPS, flagellin and pseudobactin. However, our results in Arabidopsis, bean and tomato, together with previous work on radish and carnation, show that the ability of WCS358 to induce resistance differs in different plant species. Not all plant species recognize and respond to all determinants of WCS358.

**EXPERIMENTAL PROCEDURES**

**Pathogens**

*Pseudomonas syringae* pv. *tomato* DC3000 (Whalen et al., 1991) was cultured overnight in liquid King’s medium B (KB) for 24 h at 28 °C. Cells were collected by centrifugation and resuspended in 10 mM MgSO₄, to a final density of 2.5 × 10⁷ c.f.u./mL.

Inoculum of *Colletotrichum lindemuthianum* isolate 06/038 (race 385) was prepared as described by Bigirimana and Höfte (2001). Inoculum concentration was adjusted to 10⁴ spores/mL for inoculation. *Botrytis cinerea* isolate R16, resulting from the cross SAS56 × SAS405 (Faretra and Pollastro, 1991), was maintained on potato dextrose agar medium (PDA) at 24 °C under UV light in a light/dark cycle of 12/12 h. Spores of 10-day-old cultures were suspended in 0.01% Tween 20 and filtered, and the conidial concentration was determined with a haemocytometer. The conidial suspension was diluted in a solution of 0.01 M glucose and 6.7 mM KH₂PO₄ to a final concentration of 10⁵ conidia/mL (Audenaert et al., 2002a; De Meyer and Höfte, 1997).

**Biocontrol strains and growth conditions**

*Pseudomonas putida* WCS358 was maintained on KB agar plates (King et al., 1954). Bacterial determinants involved in ISR by this strain were investigated using Tn5 transposon mutant JM218, which is defective in biosynthesis of the fluorescent siderophore pseudobactin (PB-) (Leeman et al., 1995; Marugg et al., 1985), non-motile Tn5 mutant GMB6 lacking flagella (Fla-) (de Groot et al., 1996), and a spontaneous phase-resistant mutant (43b) lacking the O-antigenic side chain of the lipopolysaccharide (OA-) (De Weger et al., 1989). Strains were grown on KB agar plates for 24 h at 28 °C, suspended in 10 mM MgSO₄ and collected by centrifugation.
Extraction of bacterial determinants from WCS358

Pseudobactin preparations were isolated from culture supernatant of strain WCS358 grown in 2 L of standard succinate medium (SSM) (pH 7, 48 h, 27 °C), containing 5.10^5 c.f.u./mL. Cell-free culture supernatant was lyophilized, and pseudobactin was purified as described by Raaijmakers et al. (1994). The yield was 302 mg with a purity of approximately 95%, as judged by its molar extinction coefficient of 13 670 at 400 nm and pH 7. Taking into account a loss of 50% in the purification procedure, the pseudobactin yield equates to 5 × 10^12 cells.

Lipopolysaccharide-containing cell walls were isolated from bacterial cultures on five overgrown KB agar plates (overnight at 28 °C), equating to 5 × 10^11 cells. Bacterial cells were suspended in phosphate-buffered saline (10 mM, pH 7.2), centrifuged (4000 g, 5 min) and lyophilized. Lyophilized cells (100 mg) were resuspended in 10 mL of 50 mM Tris/HCl, 2 mM EDTA buffer (pH 8.5) and sonicated six times at resonance amplitude for 15 s at 0–4 °C. The sonicated suspension was centrifuged (6000 g, 20 min) to remove intact cells, and the supernatant was again centrifuged (8000 g, 60 min) to obtain the crude LPS (13.3 mg) as a pellet. Taking into account a loss of 50% during the isolation, the amount of crude LPS equates to 2.5 × 10^11 cells. The pellet was stored at −20 °C (Leeman et al., 1995).

Flagella were prepared from bacterial cultures on four overgrown KB agar plates (overnight at 28 °C), equating to 4 × 10^11 cells. Bacterial cells were suspended in 20 mM K-phosphate buffer, pH 7.4 (KP buffer), and centrifuged (5700 g, 5 min). The pellet was resuspended in KP buffer, and treated in a blender (Ultra-Turrax T25, Janke & Kurkel IKA Labortechnik) at 21 000 g to shear off the flagella. Intact cells and cell debris were removed by centrifugation (23 400 g, 1 h, 4 °C). The supernatant was centrifuged in an ultracentrifuge (60 000 g, 4 h, 4 °C) to obtain the flagella in the pellet (16.7 mg). Taking into account a loss of 50% in the purification procedure, the amount of isolated flagella equates to 2 × 10^11 cells. The pellet was stored at −20 °C.

Plant material and experimental set-up

Experiments with Arabidopsis were carried out as described by Van Wees et al. (1997). Seeds of A. thaliana accession Col-0 were sown in river sand, and seedlings were grown in a growth chamber with an 8-h day and 16-h night cycle at 24 and 20 °C, respectively, and at 70% relative humidity. After 2 weeks, the seedlings were transferred to a potting soil–sand mixture, which had been autoclaved twice for 1 h on alternate days. Strain WCS358 or its mutants were mixed through the autoclaved soil to a final density of 5 × 10^7 c.f.u./g soil. For the treatments with bacterial components, material isolated from 5 × 10^9 bacterial cells was mixed with (per gram of soil) 2.7 µg cell-wall preparation, 4.18 µg flagella preparation and 3.02 µg pseudobactin.

Before sowing, bean seeds (Phaseolus vulgaris L. cv. Prelude Royal) or tomato seeds (Lycopersicon esculentum L. cv. Money-maker) were soaked in a bacterial suspension of the biocontrol strain, or water as a control, for 5 min. Bean or tomato seeds treated with bacteria or water were sown in a potting compost soil. The soil was mixed with bacterial inoculum to a concentration of 10^9 c.f.u./g. In the control treatment soil was treated with sterile demineralized water (Audenaert et al., 2002b; Bigirimana and Höfte, 2002). Seven days after sowing, plants and soil were treated a second time by pouring 30 mL of a bacterial suspension of 10^8 c.f.u./mL on top of the soil. Bean plants were grown for 12 days and tomato plants for 5 weeks under greenhouse conditions (24 °C with a 16-h photoperiod and high humidity).

For testing bacterial components, seeds were germinated on sterile wet filter paper for about 1 week. The bacterial compounds were dissolved in ultra pure water at a final concentration equivalent to 10^6 c.f.u./mL (for pseudobactin: 6.04 µg/mL; for LPS-containing cell-wall preparations: 5.3 µg/mL; for flagella: 8.35 µg/mL). Roots were immersed in 200 mL of a solution of one of the bacterial determinants for 2 min and transferred to soil.

Bioassays for induced resistance

Bacteria and control-treated Arabidopsis plants were challenged when 5 weeks old by dipping the leaves in a suspension of P. syringae pv. tomato DC3000 (Whalen et al., 1991) containing 2.5 × 10^7 c.f.u./mL in 10 mM MgSO4 supplemented with 0.01% (v/v) Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands). The plants were placed at 100% relative humidity. Arabidopsis plants were inoculated by the dip inoculation method as used and described by Pieterse et al. (1996), Pieterse et al. (1998) and Van Wees et al. (1997). For each treatment, 25 plants were used and 3 or 4 days after inoculation, all inoculated leaves per plant were scored (between 20 and 30 leaves). Leaves showing necrotic lesions surrounded by chlorosis were scored as diseased (Pieterse et al., 1996). The experiments were repeated three times with similar results. Data were statistically analysed for significance using one-way analysis of variance (ANOVA), followed by Fisher’s least significant differences test (P = 0.05).

Detached primary leaves of 12-day-old bean plants were inoculated with Colletotrichum lindemuthianum as described by Bigirimana and Höfte (2001). Each experiment was carried out twice with 12 leaves per treatment. Results of a representative experiment are shown. Anthracnose severity (Fig. 4A) was evaluated 7 days after inoculation using a scale of 1–9, based on the percentage of leaf surface covered by necrotic lesions, as described by Bigirimana and Höfte (2001): (1) no visible anthracnose symptoms, (3) leaf with a few isolated small lesions covering approximately 1% of the total area, (5) large lesions covering approximately 5% of the leaf area, (7) large lesions covering approximately 10% of the area, (9) large lesions covering 25% or more of the leaf area.
Data were statistically analysed using a Kruskal–Wallis multiple comparison test completed by a Mann–Whitney comparison test at $P = 0.05$.

Primary leaves of bean or tertiary leaves of tomato plants were inoculated with $B. \text{cinerea}$ isolate R16 (Faretra and Pollastro, 1991) by the detached leaf method as described by Audenaert et al. (2002a). Each tomato leaf was inoculated with ten droplets of 5 $\mu$L of conidial suspension, whereas each bean leaf was inoculated with ten droplets of 10 $\mu$L. Disease development was evaluated 5 days later by recording numbers of spreading and non-spreading lesions (Fig. 4A). Each experiment was carried out at least twice with ten leaves (from ten different plants) per treatment. Results of a representative experiment are shown. Data were statistically analysed by logistic regression as described (Audenaert et al., 2002a).

**Colonization of bean and tomato rhizosphere by Pseudomonas strains**

To determine rhizosphere population densities of $P. \text{putida}$ WCS358 and its mutants, roots of five plants were harvested, weighed, rinsed and homogenized in water. Serial dilutions were plated on KB medium for the parental strain, and KB agar supplemented with kanamycin (100 $\mu$g/mL) for the WCS358 mutants defective in one of the determinants. After overnight incubation at 28 °C, the number of colony-forming units per gram of root was determined. Wild-type WCS358 colonies were fluorescent on KB medium after re-isolation from the roots and could be clearly distinguished from non-fluorescent colonies that were present on roots of control plants. On KB supplemented with kanamycin no growth was observed after plating suspensions from control roots.

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**REFERENCES**


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