

Role of iron-regulated metabolites in *Arabidopsis* root colonization by *Pseudomonas fluorescens* WCS374

M. Djavaheri¹, J. Mercado-Blanco², L.C. Van Loon¹ & P.A.H.M. Bakker¹

¹Department of Biology, Section Phytopathology, Utrecht University, P.O. Box 80084, 3508 TB Utrecht, The Netherlands;

²Departamento de Proteccion de Cultivos, Instituto de Agricultura Sostenible (CSIC), 14080 Cordoba, Spain

Abstract: The plant growth-promoting rhizobacterium *Pseudomonas fluorescens* WCS374 produces several iron-regulated metabolites, including the fluorescent siderophore pseudobactin, salicylic acid (SA), and pseudomonine, a siderophore that contains a SA moiety. To study the functional role of pseudomonine and SA in the colonization of roots of *Arabidopsis thaliana*, genes *pmsA* and *pmsB* of the pseudomonine biosynthesis gene cluster were disrupted using homologous recombination. These mutants, defective in SA and pseudomonine biosynthesis, were further subjected to Tn5 mutagenesis to generate mutants also deficient in pseudobactin production. The resulting double mutants still appeared to have siderophore activity. The SA/pseudomonine and the double mutants colonized *Arabidopsis* roots to the same extent as the wild type bacterium. Surprisingly, a mutant that produced SA and pseudomonine but lacked pseudobactin colonized the plant roots significantly better. Under the conditions tested production of iron-regulated metabolites does not seem necessary for effective colonization of the *Arabidopsis* rhizosphere by WCS374.

Key words: *Pseudomonas fluorescens*, Pseudobactin, Root colonization, Salicylic acid, Siderophore

Introduction

Under conditions of iron limitation, microorganisms produce siderophores that sequester iron from the environment and subsequently transport it into the microbial cell. When iron becomes limited, the rhizobacterium *Pseudomonas fluorescens* strain WCS374 produces the fluorescent siderophore pseudobactin, salicylic acid (SA), and pseudomonine, a siderophore that contains a SA moiety (Mercado-Blanco *et al.*, 2001). Strain WCS374 can induce systemic resistance in radish (Leeman *et al.*, 1995) but not in *Arabidopsis thaliana* (Van Wees *et al.*, 1997), whereas exogenously applied SA induces resistance in both plant species. Compared to other bacteria that have been described to produce SA, WCS374 produces high amounts *in vitro* (Leeman *et al.*, 1996), but it is not clear if it releases SA in the rhizosphere or not. Since application of exogenous SA induces resistance, the inability of WCS374 to induce resistance in *Arabidopsis* suggests that SA is not released into the rhizosphere. Instead, it might all be channeled into pseudomonine. Therefore, a study was undertaken to determine the conditions in which SA and pseudomonine are produced in the rhizosphere.

Effective root colonization by *Pseudomonas* spp. is a prerequisite for successful suppression of soil-borne plant diseases. A minimum bacterial cell density is required for both microbial antagonism and the induction of systemic resistance (Raaijmakers *et al.*, 1995). Moreover, time is required for the bacterium to express its disease-suppressive properties. Specific mutants of *P. fluorescens* WCS374 were generated to study the role of iron-regulated metabolites in the ecology of the bacterium and its ability to induce systemic resistance. Here, we report observations on the importance of these metabolites for the ability of WCS374 to colonize *Arabidopsis* roots.

Materials and methods

Bacterial strains, growth conditions and determination of SA

P. fluorescens WCS374 was originally isolated from the rhizosphere of potato (Geels and Schippers, 1983). A Tn5 insertion mutant of this strain that lacks pseudobactin production was described by Weisbeek *et al.* (1986). For the colonization studies strains were grown on King's medium B agar plates for 24 h at 28°C. Bacterial cells were scraped off the plates and suspended in 10 mM MgSO₄. For determination of SA production WCS374 was grown in liquid standard succinate medium (SSM) for 48 h. Culture supernatants were extracted with chloroform as described before (Mercado-Blanco *et al.*, 2001). SA was quantified by measuring the absorbance of the deep purple Fe³⁺-SA complex at 527 nm.

Arrangement of pseudomonine biosynthesis and transport genes in WCS374

An *Eco*RI fragment cloned in plasmids pE1R and pE4R that contains 15.795 bp of the 28-kb fragment from WCS374 responsible for SA and pseudomonine biosynthesis and transport was sequenced following a primer-walking strategy. Sequencing was performed in both directions. Sequences were edited using DNASTAR (Lasergene) software. Homology analysis was done with the BLAST program at the NCBI network service.

SA biosynthetic mutants of WCS374

We employed homologous recombination to knock out either gene *pmsA*, which codes for histidine decarboxylase, or gene *pmsB*, which codes for isochorismate-pyruvate lyase. A kanamycin resistance cassette from plasmid pUC4K was introduced within these genes and the disrupted genes were cloned in the suicide plasmid pSUP202. The recombinant plasmids were used for homologous recombination with WCS374.

Mutants defective in the production of pseudobactin

The *pmsA* and *pmsB* deficient mutants were subjected to Tn5 mutagenesis to isolate mutants additionally defective in pseudobactin production. The mobilization system of *Escherichia coli* strain S17-1 and the suicide plasmid pJQ18, which carries a modified Tn5 (Mob Tc^r), were used.

Determination of iron-chelating capacity

Iron-chelating capacity of the bacteria was quantified by relative halo size around colonies grown on Chrome Azurol S (CAS) medium, which is a universal medium for the determination of siderophore activity. Production of siderophore leads to a shift in colour from blue (chelated CAS) to orange (free CAS).

Colonization of Arabidopsis roots by WCS374 and its siderophore mutants

Seedlings of *A. thaliana* Col-0 were grown in sterile sand for two weeks. Three plantlets were transplanted into 60-ml pots containing a mixture of sand and potting soil that was autoclaved twice for 1 h with a one-day interval. Before transferring the plantlets, the potting soil was supplemented with a suspension of bacteria to a final density of 10³ cfu/gram soil. Plants were watered on alternate days and once a week supplied with a modified half-strength Hoagland nutrient solution without iron. Roots were sampled and the numbers of bacteria per gram of root were determined by plating serial dilutions of rhizosphere suspensions in 0.01M MgSO₄ on media with appropriate antibiotics one, two and three weeks after transplanting.

Results and discussion

SA and pseudomonine biosynthesis genes in WCS374

The SA and pseudomonine biosynthesis genes in WCS374 are organized in a cluster designated *pmsCEAB* (Figure 1A), containing two ORFs (*pmsB* and *C*) with homology to

chorismate-utilizing enzymes, one (*pmsE*) with homology to enzymes involved in siderophore biosynthesis, and one (*pmsA*) encoding a putative histidine decarboxylase (Mercado-Blanco *et al.*, 2001).

Additional ORFs involved in pseudomonine biosynthesis and transport have now been identified (Figure 1B). Three ORFs (1-3) may complete the pseudomonine biosynthesis machinery, together with the previously characterized *pmsCEAB* genes. Additional ORFs (5-10) were identified that may be involved in transport and uptake of pseudomonine.

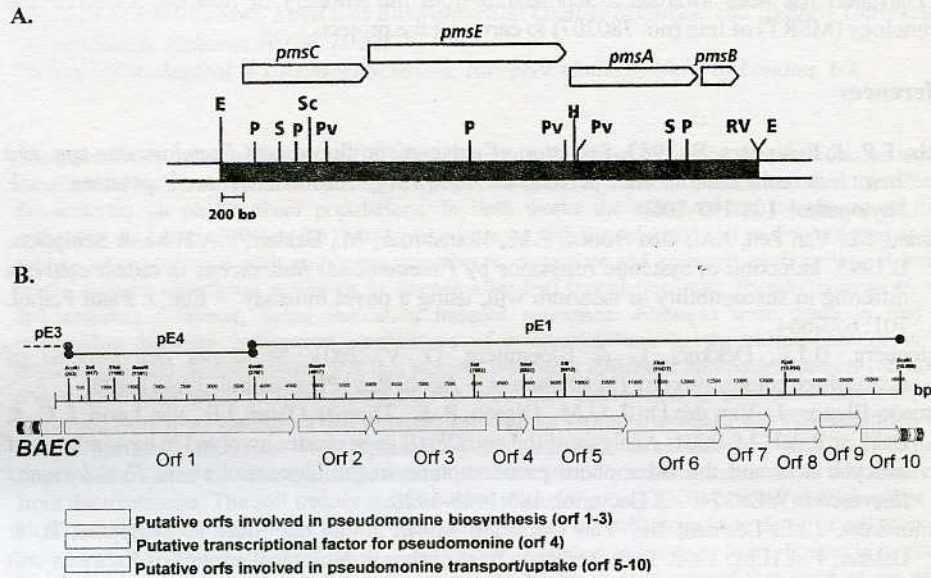


Figure 1. Salicylic acid (A) and pseudomonine biosynthesis and transport coding region (B) in *Pseudomonas fluorescens* WCS374.

Salicylate and double mutants

Kanamycin (Km)-resistant, tetracyclin (Tc)-sensitive colonies were selected in a two-step screening procedure. Presence of a unique insert within the disrupted genes was confirmed using primer pairs SAL01/SAL02 and SAL02/HDC01, which amplified the *pmsB* and *pmsAB* regions of the genome (Mercado-Blanco *et al.*, 2001), respectively. The mutants no longer produced SA in iron-limiting liquid SSM, as determined by chloroform extraction. Tc-resistant colonies were obtained after conjugation of the SA/pseudomonine mutants with *E. coli* S17-1 carrying the Tn5 transposon. Colonies that were non-fluorescent on King's medium B, indicating that pseudobactin production was abolished, were selected. These double mutants still had siderophore activity as measured on CAS medium. This observation suggests the presence of yet another molecule with siderophore activity in WCS374.

Colonization of *Arabidopsis* roots by WCS374 and its mutants

All mutants colonized *Arabidopsis* roots to levels similar to the wild type. Surprisingly mutants that lack pseudobactin but still produce SA and pseudomonine, reached up to 10-fold

higher cell densities. Population densities of WCS374, SA and double mutants increased to about 10^6 cfu/g root within the first week after transplanting the seedlings. Thus, iron-regulated metabolites do not seem to contribute to effective colonization of the *Arabidopsis* rhizosphere by WCS374. Pseudobactin production even seems to reduce root colonizing capacity. The mutants are currently used to investigate the role of the iron-regulated metabolites of WCS374 in the induction of systemic resistance in *Arabidopsis* and radish.

Acknowledgements

M. Djavaheri has been awarded a scholarship from the Ministry of Science, Research and Technology (MSRT) of Iran (no. 780207) to carry out the project.

References

- Geels, F.P. & Schippers, B. 1983. Selection of antagonistic fluorescent *Pseudomonas* spp. and their root colonization and persistence following treatment of seed potatoes. – J. Phytopathol. 108:193-206.
- Leeman, M., Van Pelt, J.A., Den Ouden, F.M., Heinsbroek, M., Bakker, P.A.H.M. & Schippers, B. 1995. Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to fusarium wilt, using a novel bioassay. – Eur. J. Plant Pathol. 101: 655-664.
- Lugtenberg, B.J.J., Dekkers, L. & Bloemberg, G. V. 2001. Molecular determinants of rhizosphere colonization by *Pseudomonas*. – Annu. Rev. Phytopathol. 39:461-490.
- Mercado-Blanco, J., Van der Drift, G.M., Olsson, P. E., Thomas-Oates, J.E., van Loon, L.C. & Bakker, P.A.H.M. 2001. Analysis of the *pmsCEAB* gene cluster involved in biosynthesis of salicylic acid and the siderophore pseudomonine in the biocontrol strain *Pseudomonas fluorescens* WCS374. – J. Bacteriol. 183:1909-1920.
- Raaijmakers, J.M., Leeman, M., Van Oorschot, M.M.P., Van der Sluis I., Schippers, B. & Bakker, P.A.H.M. 1995. Dose-response relationship in biological control of fusarium wilt of radish by *Pseudomonas* spp. – Phytopathology. 85: 1075-1081.
- Van Wees S.C.M., Pieterse, C.M.J., Tryssenaar, A., Van t Westende, Y.A.M., Hartog, F. & Van Loon, L.C. 1997. Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. Mol. – Plant-Microbe Interact. 10: 716-724.
- Weisbeek, P.J., Van der Hofstad, G.A.J.M., Schippers, B. & Marugg, J.D., 1986. Genetic analysis of the iron-uptake system of two plant growth-promoting *Pseudomonas* strains. – In: Swinburne, T.R. (Ed.): Iron, Siderophores and Plant Diseases. Plenum Press, New York: 299-313.