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Molecular analysis of iron transport in plant growth-promoting *Pseudomonas putida* WCS358

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Summary. Root-colonizing *Pseudomonas putida* WCS358 enhances growth of potato in part by producing under iron-limiting conditions a yellow-green, fluorescent siderophore designated pseudobactin 358. This siderophore efficiently complexes iron(III) in the rhizosphere, making it less available to certain endemic microorganisms, including phytopathogens, thus inhibiting their growth. At least 15 genes distributed over five gene clusters are required for the biosynthesis of pseudobactin 358. High-affinity iron(III) transport in strain WCS358 is initiated by an 86-kDa outer membrane receptor protein (PupA) which appears to be specific for ferric pseudobactin 358. PupA shares strong similarity with TonB-dependent receptor proteins of *Escherichia coli*, which suggests a TonB-like protein in strain WCS358 is required for iron(III) transport. Strain WCS358 possesses a second uptake system for ferric pseudobactin 358 and structurally diverse ferric siderophores produced by other microorganisms. A second receptor gene (*pupB*) responsible for iron transport from pseudobactin BN7 or pseudobactin BN8 has been identified. The production of this and certain other ferric siderophore receptor proteins requires that strain WCS358 be grown in the presence of these siderophores. An apparent regulatory gene required for the expression of *pupB* is located adjacent to *pupB*. Two positive regulatory genes have been identified which can independently activate, under low-iron(III) conditions, transcription of genes coding for the biosynthesis of pseudobactin 358.

Key words: Iron transport – Siderophores – *Pseudomonas putida* – Genetics – Receptors

Introduction

Specific strains of the *Pseudomonas fluorescens/Pseudomonas putida* group rapidly colonize plant roots of several crops and cause statistically significant yield increases (Burr and Caesor 1984; Kloepper et al. 1989). Enhanced plant growth caused by these strains is often accompanied by reductions in root-zone populations of fungi and bacteria. These beneficial strains are also effective biocontrol agents of certain soil-borne fungal pathogens (Burr and Caesor 1984; Kloepper et al. 1989). These fluorescent pseudomonads exert their beneficial effects in part by producing under iron-limiting conditions yellow-green, fluorescent siderophores (microbial iron transport agents) (Leong 1986; Schippers et al. 1987). These siderophores efficiently complex environmental iron, making it less available to certain endemic microorganisms, including phytopathogens, thus inhibiting their growth. This growth inhibition is probably due to their lack of an iron transport system for these ferric siderophores.

We describe in this article the use of molecular genetic techniques to study high-affinity iron(III) transport in plant growth-promoting *Pseudomonas putida* WCS358.

Pseudobactin 358

The structure of pseudobactin 358, the siderophore produced by *P. putida* WCS358 has recently been elucidated (GAJM van der Hofstad, personal communication) and is given in Fig. 1. In the nonapeptide the α-hydroxyaspartic acid and the N^{δ}-hydroxyornithine constitute, together with the *o*-dihydroxy-aromatic group derived from the quinoline derivative, the three bidentate iron(III)-chelating groups. The structure of pseudobactin 358 very much resembles the other fluorescent siderophores (Leong 1986; Persmark et al. 1990, and references therein). These structural differences in fluorescent siderophores are believed to be important in iron uptake since outer membrane receptor proteins for ferric siderophores appear to recognize specific features of the ferric siderophore. Pseudobactin 358 could be utilized by only a few of several hundred rhizosphere fluorescent pseudomonads that were tested.

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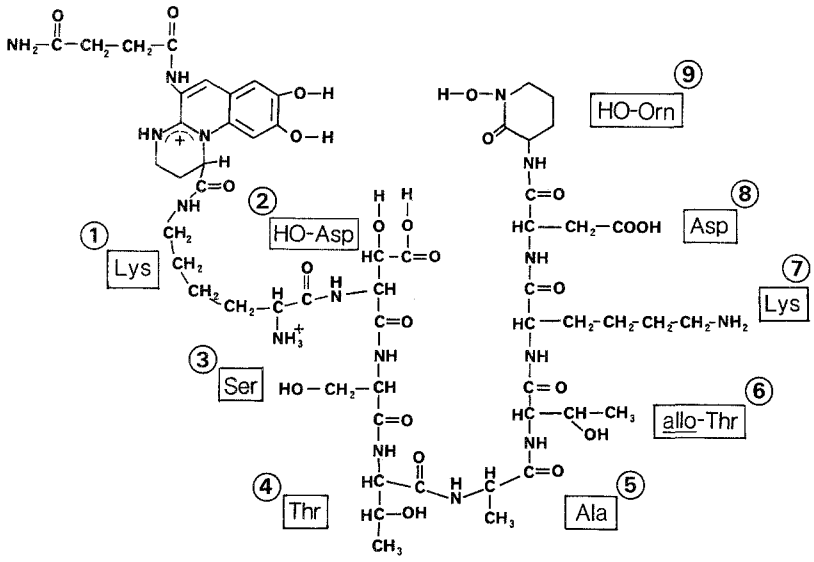

Pseudobactin 358

Fig. 1. Proposed chemical structure of pseudobactin 358, the siderophore of *Pseudomonas putida* WCS358

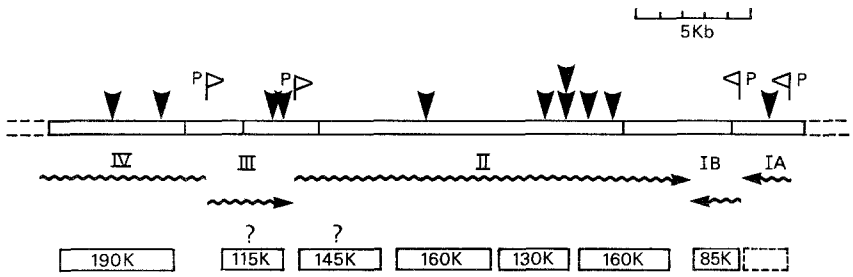

Fig. 2. Genetic organization of cluster A. The localization and orientation of the transcriptional units (IA, IB, II, III, IV) are indicated by the wavy lines. Arrowheads on the *Eco*RI restriction map represent Tn*5* insertions in the siderophore-defective mutants. Flags (with the letter P) represent possible promoter regions (190 K ≡ 190 kDa). The proposed location and the sizes of the polypeptide products are indicated by the closed boxes in the lower part of the figure. The size of the protein represented by the hatched box is unknown

The biosynthesis of pseudobactin 358 requires at least 15 genes distributed over five gene clusters (Marugg et al. 1988). This minimum number of genes seems reasonable considering the structural complexity of pseudobactin 358 and the fact that only a fraction of the non-fluorescent mutants could be complemented. In a similar way Moores et al. (1984), identified a minimum of 12 genes arranged in four clusters that were required for biosynthesis of pseudobactin, the native siderophore of plant growth-promoting *Pseudomonas* B10.

A major gene cluster (A) required for pseudobactin 358 biosynthesis in strain WCS358 consists of at least five transcriptional units (IA, IB, II, III, IV; Fig. 2), some of which are relatively large (Marugg et al. 1988). The polypeptides that are encoded by these transcriptional units were determined by analysis of *E. coli* minicells. The iron-regulated expression of genes in transcripts IB, II, and III appears to be at the level of transcription.

With the exception of the 85-kDa protein, which is probably the outer membrane receptor protein for ferric pseudobactin 358 (see following section), the biosynthetic functions of genes in this cluster are not known. However, three mutants in this region synthesized mutated non-fluorescent siderophores that contained the complete wild-type peptide (GAJM van der Hofstad, personal communication). These results, together with the overall structure of the siderophore, i.e. a short peptide chain attached to a fluorescing group, suggest a biosynthetic pathway in which the biosynthesis of the fluorescing group is preceded by the enzymatic synthesis of the peptide part and that specific mutants in cluster A may be defective in certain steps of the synthesis of the hydroxyquinoline derivative.

Iron uptake and ferric siderophore receptors

High-affinity iron transport involving pseudobactin 358 in strain WCS358 is initiated by an iron-regulated 86-kDa outer membrane receptor protein designated PupA, which appears to be specific for ferric pseudobactin 358 (Marugg et al. 1989; Bitter et al. 1991). The PupA protein is responsible for at least 70% of the transport of ferric pseudobactin 358 (Bitter et al. 1991).

The gene encoding PupA has been identified and sequenced. The mature protein consists of 772 amino acids (86.01 kDa) with a signal sequence of 47 amino acids, which is extremely long for prokaryotes (Bitter et al. 1991). The receptor protein shares strong similarity with four regions of TonB-dependent receptor proteins of *E. coli*, including BtuB for vitamin B_{12} (Heller and Kadner 1985), FecA for ferric dicitrate (Pressler et al. 1988), FepA for ferric enterobactin (Lundrigan and Kadner 1986), FhuA for ferrichrome (Coulton et al. 1986), IutA for ferric aerobactin (Krone et al. 1985), FhuE for ferric coprogen, ferrioxamine B, and ferric rhodotorulic acid (Sauer et al. 1990), and Cir for colicin I (Nau and Konisky 1989). The TonB protein is presumed to be an inner membrane protein which energizes outer membrane receptor proteins (Hantke and Braun 1975). The fact that PupA contains the regions of similarity suggests the presence of a TonB-like protein with a similar energy coupling system in *P. putida*. This TonB coupling system could be a universal prokaryotic system for active transport across the outer membrane.

The amino acid sequence of PupA is most related to that of TonB-dependent FhuE; an alignment of these two sequences showed 259 perfect matches, which results in 31.4% similarity (Bitter et al. 1991). The observed similarity suggests that PupA and FhuE are evolutionarily and/or functionally related. FhuE however is a receptor for hydroxamate type of siderophores, whereas PupA seems to be a specific receptor for pseudobactin 358, which contains both an hydroxamate and a catechol group. The conserved regions could be transmembrane domains because (a) the similarity is concentrated in regions with regular intervals, and (b) transmembrane domains are the most conserved regions in related outer membrane proteins (Tommassen 1988).

P. putida WCS358 possesses a unique ability to assimilate iron(III) via many structurally diverse siderophores, which are produced by other microorganisms (Fig. 3). Strain MH100, which is a chromosomal mutant of strain WCS358 containing a Tn*5* insertion in the *pupA* gene, no longer produced the 85-kDa iron-regulated outer membrane receptor protein (Bitter et al. 1991). Nevertheless, the ability of this mutant to acquire iron(III) from exogenous siderophores was virtually unaffected, although its utilization of ferric pseudobactin 358 was reduced by 70% (Fig. 3). In contrast, no transport of Fe(III) via pseudobactin was observed in *Pseudomonas* B10 Tn*5* mutants lacking the 85-kDa outer membrane receptor protein for ferric pseudobactin (Magazin et al. 1986). These results suggest that PupA is a specific receptor for ferric pseudobactin 358 and that an alternative iron uptake system recognizes ferric pseudobactin 358 and heterologous ferric siderophores.

A second receptor gene designated *pupB* has been identified from a pLAFR1 gene bank of WCS358 in a manner analogous to the *pupA* gene (Marugg et al. 1989). Introduction of this gene located on pMK15 into heterologous *Pseudomonas* A124 or *Pseudomonas fluorescens* WCS374 rendered these strains no longer susceptible to iron starvation by pseudobactin BN7, the native siderophore of *Pseudomonas* BN7. In addition, strain A124 or strain WCS374 harboring pMK15 became able to transport ferric pseudobactin BN8 produced by *Pseudomonas* BN8. Pseudobactin BN7 is structurally distinct from pseudobactin BN8 (W. Bitter, unpublished results).

The PupB protein was identified as follows. Strain A124 harboring pMK15 did not exhibit any additional outer membrane proteins (Fig. 4, lane 6) than strain A124 (Fig. 4, lane 5). However, when strain A124 (pMK15) was grown in the presence of 40 μM pseudo-

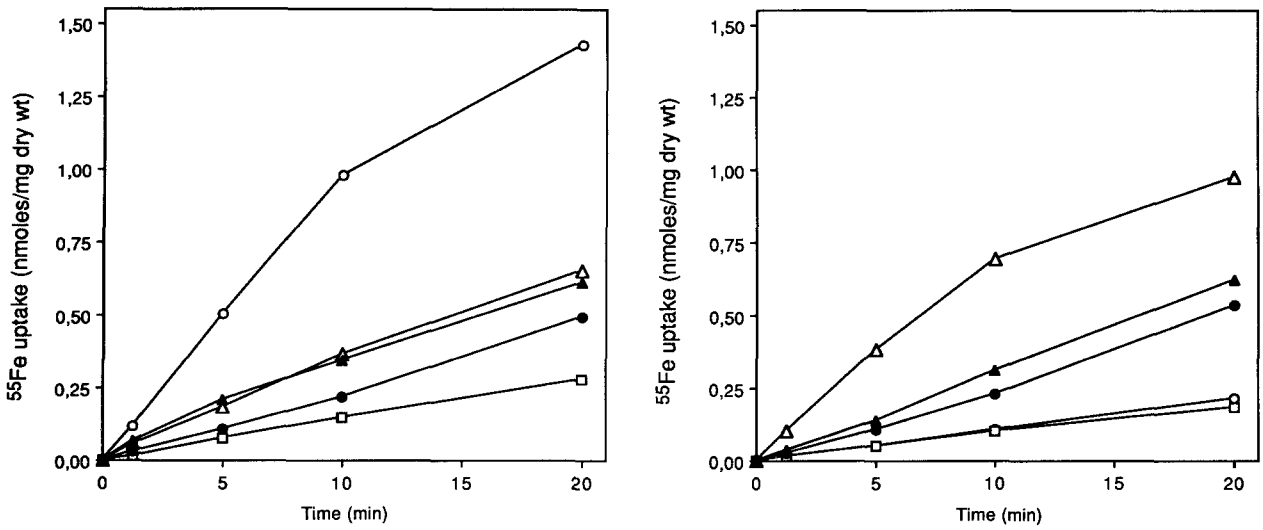

Fig. 3. Pseudobactin mediated $^{55}Fe^{3+}$ transport by cells of WCS358 (left) and MH100 (right) grown under iron limitation. Uptake of five different ferric pseudobactins was analyzed: pseudobactin 358 (○), pseudobactin 374 (△), pseudobactin BN2 (□), pseudobactin BN7 (▲), and pseudobactin BN8 (●)

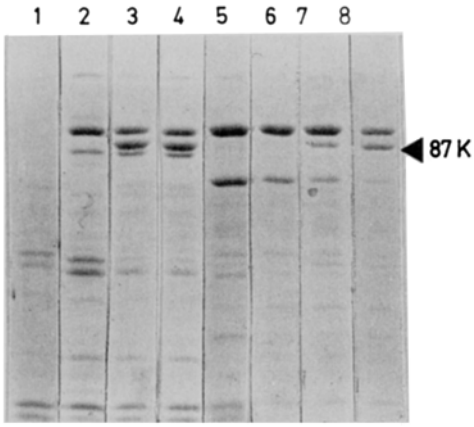

Fig. 4. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of cell envelope preparations of cells grown in KB medium with various supplements. Lanes: 1, WCS358, 50 μM $FeCl_3$; 2, WCS358; 3, WCS358, 40 μM pseudobactin BN7; 4, WCS358, 40 μM pseudobactin BN8; 5, A124; 6, A124 (pMK15); 7, A124 (pMK15), 40 μM pseudobactin BN7; 8, A124 (pMK15), 40 μM pseudobactin BN8. The arrow indicates the 87-kDa protein

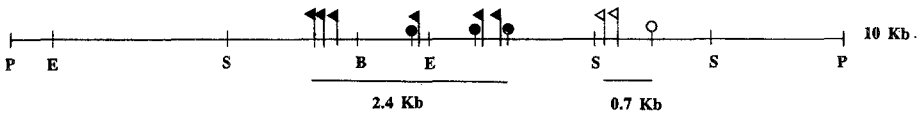

Fig. 5. Location of Tn*5* (○, ●) and Tn*3*HoHo*1* (△, ▲) insertions in the 10-kb *Pst*I insert of pRJ1, which cause a loss of the ability to make A124 cells competent for pseudobactin BN7 utilization. Arrows show the direction of Tn*3*HoHo*1* insertions. Horizontal lines denote the location and minimum size of the two transcriptional units. Transposon insertions in region I are indicated by closed symbols and those in region II by open symbols. B, *Bgl*II; P, *Pst*I; E, *Eco*RI; S, *Sma*I

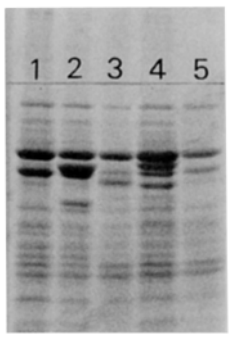

Fig. 6. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of cell envelope preparations of WCS358 cells grown in KB medium (lane 1); and supplemented with 40 μM pseudobactin BN7 (lane 2); 40 μM pseudobactin B10 (lane 3); 40 μM pyoverdine (lane 4); 40 μM deferriferrichrome A (lane 5)

bactin BN7 or pseudobactin BN8, an additional iron-regulated outer membrane protein of 87 kDa was induced (Fig. 4, lanes 7 and 8, respectively). Under iron-limitation, strain WCS358 produces two outer membrane proteins of 85 kDa (PupA) and 88 kDa, whose function is unknown (Fig. 4, lanes 1 and 2, respectively). Growth of strain WCS358 in the presence of pseudobactin BN7 or pseudobactin BN8 induced the same 87-kDa protein (Fig. 4, lanes 3 and 4, respectively). The 87-kDa protein was confirmed as the *pupB* gene product by constructing chromosomal mutants of strain WCS358 with transposon insertions in *pupB*. Such mutants were generated as follows.

Transposon Tn*5* and Tn*3*HoHo*1* mutagenesis of pMK15 and pRJ1, containing a 10-kb *Pst*I subclone, delineated the DNA region, which made strain A124 competent for the utilization of ferric pseudobactin BN7 (Fig. 5). Transposon mutations within this region were marker exchanged onto the chromosome of strain WCS358; the resulting genomic mutants no longer produced the 87-kDa protein. By complementation analysis, these mutants were divided into two complementation groups. The mutants on the left defined a genetic region (I) of at least 2.4 kb, whereas the mutants on the right delineated a locus (II) of at least 0.7 kb. Region I probably encodes the *pupB* structural gene since its nucleotide sequence is significantly similar to that of *pupA*. Region II is probably involved in the induction of the *pupB* gene. Both regions are transcribed from right to left as determined by Tn*3*HoHo*1* mutagenesis.

Although the above transposon mutants of strain WCS358 no longer produced the 87-kDa protein, they were still capable of iron(III) assimilation via pseudobactin BN7 or pseudobactin BN8, although with decreased efficiency. This suggests that strain WCS358 possesses a second uptake system for ferric pseudobactin BN7 and ferric pseudobactin BN8.

Growth of strain WCS358 in the presence of pseudobactin B10 (Teintze et al. 1981) or pyoverdine (Briskot et al. 1989) but not in the presence of deferriferrichrome A resulted in the induction of specific outer membrane proteins (Fig. 6). This suggests that iron transport via heterologous siderophores in strain WCS358 is mediated by several different receptor proteins and that the production of some of these receptors is induced by growth of strain WCS358 in the presence of specific siderophores.

Regulation of iron transport

Pseudobactin 358 and PupA are produced by strain WCS358 only under iron-limitation. Several operons

coding for pseudobactin 358 biosynthesis in gene cluster A are regulated by iron(III) at the transcriptional level. A deletion analysis of the siderophore promoter of transcript III in cluster A revealed the 73-bp region upstream of the transcriptional start was required for proper iron-regulated expression. This region could be the binding site for a positive regulatory protein; this information was exploited to clone such proteins.

Broad-host range pMK14 (IncQ) is a transcriptional fusion plasmid containing a 4.8-kb *Hin*dIII-*Eco*RI promoter fragment of transcript III fused to the structural gene for catechol 2,3-dioxygenase (*xylE*). This enzyme converts catechol into a yellow product, which is easily assayable. XylE activity was expressed in strain WCS358 (pMK14) only when cells were grown under iron-limited conditions. However, the rhizosphere fluorescent pseudomonad *Pseudomonas* A225 harboring pMK14 did not exhibit XylE activity when grown under iron-limitation. We postulated that strain A225 did not possess a positive regulator capable of activating the WCS358 siderophore promoter.

When the pLAFR1 (IncP) gene bank of WCS358 was mated en masse with strain A225 (pMK14), two unique cosmid clones were identified from transconjugants which expressed XylE activity only under iron-limitation. These two putative activator genes, designated 1A and 12A, were capable of independently activating the siderophore promoter of transcript III. Transposon Tn*5* and Tn*3*HoHo*1* mutagenesis and subcloning are being performed on these two cosmids to identify the functional regions of these two activators. Siderophore biosynthesis in *Vibrio anguillarum* is also activated by two independent positive regulators (Salinas et al. 1989).

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