Identification of Bacterial Proteins Mediating the Interactions Between *Pseudomonas putida* UW4 and *Brassica napus* (Canola)

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The influence of canola root exudates on the proteome of *Pseudomonas putida* UW4 and the mutant strain *P. putida* UW4/AcdS⁻, which lacks a functional 1-aminocyclopropane-1-carboxylate deaminase gene, was examined using two-dimensional difference in-gel electrophoresis. Seventy-two proteins with significantly altered expression levels in the presence of canola root exudates were identified by mass spectrometry. Many of these proteins are involved in nutrient transport and utilization, cell envelope synthesis, and transcriptional or translational regulation and, hence, may play important roles in plant–bacterial interactions. Four proteins showing large changes in expression in response to canola root exudates in both the wild-type and mutant strains of *P. putida* UW4 (i.e., outer membrane protein F, peptide deformylase, transcription regulator Fis family protein, and a previously uncharacterized protein) were both overexpressed and disrupted in *P. putida* UW4 in an effort to better understand their functions. Functional studies of these modified strains revealed significantly enhanced or inhibited plant-growth-promoting abilities compared with the wild-type *P. putida* UW4, in agreement with the suggested involvement of three of these four proteins in plant–bacterial interactions. The work reported here suggests strategies to both identify potential antibacterial agents and develop bacterial strains that might be useful adjuncts to agriculture. This approach may be an effective means of identifying key proteins mediating the interactions of bacteria with their rhizosphere environment.

Various components in plant exudates play important roles in plant–bacterial interactions (de Weert et al. 2002; Mark et al. 2005). Most previous studies of plant exudates have focused on their ability to be utilized by surrounding bacteria and, therefore, facilitate plant–bacterial interactions via bacterial chemotaxis, root colonization, and root growth stimulation (de Weert et al. 2002; Kamilova et al. 2006; Lugtenberg et al. 1999). For example, Mark and associates (2005) investigated global influences of plant exudates on bacterial gene expression and identified genes involved in rhizosphere colonization using microarrays, including some genes that had not been characterized previously. Most proteomic studies of plant–bacterial

interactions have focused on either symbiotic nitrogen fixing (Djordjevic et al. 2003; Natera et al. 2000) or phytopathogenic (Bouchart et al. 2007; Caldelari et al. 2006) bacterial strains. In spite of their potential agricultural and environmental importance (Lucy et al. 2004), proteomic studies of free-living plant-growth-promoting bacteria have not been reported, in part because these organisms and their modes of action are less well characterized.

Pseudomonas putida UW4 is a plant-growth-promoting bacterium that contains the pyridoxal phosphate enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase [EC 4.1.99.4], which catalyzes the deamination of ACC to produce α -ketobutyrate and ammonia. ACC is the immediate precursor of the phytohormone ethylene, an important mediator of stress responses and plant growth and development (Abeles et al. 1992; Mattoo and Suttle 1991). A model describing how ACC deaminase-containing plant-growth-promoting bacteria modulate plant ethylene levels has been described (Glick et al. 1998). Briefly, along with other small molecules, a portion of the ACC in plants is exuded from plant roots or seed, and the bacteria attached to the surface of the seed or roots can take up some of this ACC and cleave it through the activity of ACC deaminase, thereby acting as a sink for ACC. Thus, ACC deaminase can limit inhibitory ethylene levels, often associated with various stresses, such as flooding, drought, pathogens, high salt, and the presence of organic and inorganic soil contaminants, while maintaining lower beneficial ethylene levels in plants (Stearns and Glick 2003). This model is consistent with a wide range of experimental data and is summarized in recent reviews (Glick 2005; Glick et al. 2007a and b; Lucy et al. 2004). The mutant strain *P. putida* UW4/AcdS⁻, lacking a functional *acdS* gene (encoding ACC deaminase), does not prevent ethylene inhibition of plant growth and, therefore, can only promote plant growth to a limited degree (Li et al. 2000). This limited plant-growth promotion is likely due to other mechanisms such as indole 3-acetic acid (IAA) synthesis (Li et al. 2000). Many beneficial bacteria synthesize IAA, predominantly by a tryptophan-dependent pathway, which can stimulate host root cell proliferation and elongation (Patten and Glick 1996, 2002). In addition to alleviating the inhibitory effects of ethylene, ACC deaminase-containing plant-growth-promoting bacteria also decrease the feedback inhibition of IAA signal transduction by ethylene (Glick et al. 2007a).

In the work reported here, the IAA and ACC levels in canola root exudates from control and bacteria-treated plants were compared to check the impacts of different bacterial strains on these levels. In order to identify bacterial proteins mediating interactions between the plant-growth-promoting bacterium *P. putida* UW4 and canola, protein expression profiles of bac-

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terial strains *P. putida* UW4 and *P. putida* UW4/AcdS⁻ following exposure to canola root exudates were investigated using proteomic tools.

RESULTS

Plant hormones in canola root exudates.

Canola seeds were treated with either *P. putida* UW4, *P. putida* UW4/AcdS⁻, or buffer as a control, and root exudates were collected 7 days after planting. Measurement of the IAA and ACC content of these exudates pooled from 300 plants (Fig. 1) revealed that treatment of plants with the wild-type strain dramatically increased the amount of IAA secreted by the plant whereas treatment of the AcdS⁻ mutant strain only slightly increased IAA levels. In addition, treatment of plants with the wild-type but not the AcdS⁻ mutant bacteria lowered the amount of ACC that was exuded.

Comparative protein expression profiles.

The protein expression profiles of *P. putida* UW4 and *P. putida* UW4/AcdS⁻ in response to canola root exudates were analyzed and significant changes were observed in both samples. Out of a total number of 1,757 proteins detected on the analytical gels for *P. putida* UW4, the expression levels of 220 (12.52%) proteins were significantly increased and 172 (9.79%) proteins were significantly decreased ($P \leq 0.05$, expression ratio ≥ 1.5). Results were comparable for the *P. putida* UW4/AcdS⁻ strain, although a smaller number of differentially expressed proteins were detected. For the mutant strain, 1,670 proteins were detected, where expression levels of 164 (9.82%) proteins were significantly increased and 115 (6.89%) proteins were significantly decreased. Supplementary Figure 1 shows a representative analytical gel comparing protein expression of the wild-type *P. putida* UW4 exposed to canola root exudates (red) or buffer control (green).

Mass spectrometric analyses.

In total, 72 proteins with significantly different expression levels in the presence of the added plant exudates were successfully identified by mass spectrometry via matches to homologous proteins from other *Pseudomonas* strains (Supplementary Fig. 2) and divided into nine groups based on functional annotation from the Swiss-Prot database (Fig. 2; Table 1). In Figure 2, differential expression is visualized on a scale with red indicating upregulation and green indicating downregulation for identified proteins in the presence of canola root

exudates in the wild-type and AcdS⁻ strain, respectively. White boxes represent proteins whose expression was not significantly altered. Details of the mass spectrometric identification and differential expression are included in Table 1. Sixty-four of the protein expression changes were observed in the profiles of both strains. Proteins in groups I to V were significantly upregulated in the presence of canola root exudates in both the wild type and AcdS⁻ mutant. These proteins are involved in bacterial envelope synthesis and membrane transportation, as well as metabolism of nucleic acids, amino acids, and carbohydrates. ACC deaminase was significantly upregulated in the wild type, although at only a moderate level of 1.64-fold in the wild type. On the other hand, five transcriptional regulators in group VI were downregulated while one transcription repressor NmrA-like protein was upregulated. NmrA is a negative transcriptional regulator involved in controlling nitrogen metabolite repression (Andrianopoulos et al. 1998). Protein expression levels within group VII (protein synthesis, folding, and degradation) and group VIII (oxidoreductase) exhibited both increases and decreases. Finally, group IX includes a previously uncharacterized protein, a hypothetical protein A, and cell division protein FtsA. From the 64 proteins whose expression changed significantly in both the wild type and AcdS⁻ mutant, 4 proteins were selected for additional analyses to confirm their involvement in plant-bacterial interaction.

Root elongation promoting activities of the overexpressing and knockout strains.

The first basis for the selection of the genes chosen for additional functional analyses is proteins that showed the greatest increase or decrease in expression (>7 for upregulated proteins and <-2 for downregulated proteins). Another selection criterion is confidence in the mass spectrometric identification (i.e., >90 and 80% for up- and downregulated proteins, respectively) in order to easily clone the genes from *P. putida* UW4 genome. There were four proteins that fulfilled these criteria and all of them were chosen for expression and mutagenesis studies. These proteins were one upregulated protein (outer membrane protein F) and three downregulated proteins (peptide deformylase, transcription regulator Fis family, and a previously uncharacterized protein). The genes *pdf*, *fis*, *hyp*, and *ompF* encoding peptide deformylase (Pdf), transcription regulator Fis family (Fis), uncharacterized protein (Hyp), and outer membrane protein F (OmpF), respectively, were amplified by polymerase chain reaction (PCR) using primers (Supplementary Table 2) designed based on analogues from other closely

Fig. 1. A, 1-Aminocyclopropane-1-carboxylate (ACC) and **B**, indole 3-acetic acid (IAA) concentrations in the root exudates from control and treated plants. Exudates were pooled from 300 plants, and ACC and IAA concentrations measured in triplicate analytical high-performance liquid chromatography runs.

related *Pseudomonas* strains as described below. The coding sequences of the *pdf*, *fis*, *hyp*, and *ompF* genes were determined and deposited in the GenBank database under accession numbers EU514687–EU514690.

These four genes were cloned into the *Pseudomonas*–*Escherichia* shuttle expression vector pETP constructed (Supplementary Fig. 3A) for protein expression in *P. putida* UW4. The expression of the four 6xHis-tagged recombinant proteins is shown in Figure 3A. In all instances, the expression of the cloned gene was confirmed by antibodies directed against the His-tag and from the calculated molecular mass of the proteins.

Diagnostic PCR for individual genes confirmed that the genes encoding three of these four abovementioned proteins were disrupted in the mutant strains *P. putida* UW4/Fis[−], UW4/Hyp[−], and UW4/OmpF[−]. In each case, the PCR products from the mutant strains were 1.5 kbp longer than their alleles in the wild type, reflecting the insertion of the *tetA* gene into each of these genes (Fig. 3B). The *P. putida* UW4/Pdf[−] mutant was unable to survive, presumably because this mutation was lethal.

The plant-growth-promoting ability of the wild-type, AcdS[−] mutant, four overexpressing, and three viable disruption mutant (nonexpressing) strains were tested in a canola root elongation assay (Fig. 4). This assay revealed that i) the AcdS[−] mutant demonstrated plant-growth-promoting ability to a significantly lesser extent (77.8%) than the wild type, ii) the Fis protein overexpressing and knockout strains showed no significant difference compared with the wild type, iii) the strains overexpressing the Pdf and the OmpF proteins and the Hyp protein knockout strain promoted root length more than the wild type, and iv) the ompF protein knockout strain and the Hyp protein overexpresser lost their root-length-promoting activity. Moreover, overexpression of Hyp inhibited root growth relative to the untreated plants, suggesting that high levels of this protein were detrimental to plant growth.

DISCUSSION

Both ACC and IAA play important roles in the process of plant growth promotion by bacteria (Glick 1995; Patten and Glick 2002). In agreement with a previously proposed model (Glick et al. 1998) and published data showing a decrease in endogenous ACC levels when ACC deaminase is present (Penrose and Glick 2001), treatment of plants with *P. putida* UW4 decreased the exogenous ACC content to approximately half of the control level, whereas the mutant *P. putida* UW4/AcdS[−], which doesn't have ACC deaminase, slightly increased the ACC concentration. On the other hand, there was a dramatic increase (4.5-fold) in the IAA level when plants were treated with *P. putida* UW4 and only a small increase (approximately 50%) following treatment with the mutant *P. putida* UW4/AcdS[−]. Although plant endogenous IAA and ACC levels were not measured directly, the levels found in root exudates are expected to be highly correlated to the levels in root tissues. Because ACC deaminase decreases the negative feedback of ethylene on IAA signal transduction and synthesis (Glick et al. 2007a), it is not surprising that the wild-type *P. putida* UW4 can decrease the amount of ACC and, at the same time, increase the IAA level. In fact, some plant auxin-regulated genes were upregulated following treatment with an endophytic

Fig. 2. Differentially expressed protein profiles of *Pseudomonas putida* UW4 (left column) and *P. putida* UW4/AcdS[−] (right column) in response to root exudates. Fold protein expression changes are depicted according to the color scale at the top. Protein groups and names are listed to the right.

plant-growth-promoting *P. fluorescens* strain that contains ACC deaminase (Wang et al. 2005). With the mutant strain *P. putida* UW4/AcdS⁻, the small observed increase in the IAA level may reflect exogenous IAA supplied by the bacterium without any change to the feedback inhibition of ethylene on IAA signal transduction and synthesis (Glick et al. 2007a; Li et al. 2000). The increased level of ACC following treatment of plants with the mutant is most likely a consequence of the IAA activation of ACC synthase. This data is consistent with the model describing how an ACC deaminase-containing bacterium modulates levels of both ethylene and IAA (Glick et al. 1998, 2007a). In this model, ethylene inhibition of IAA signal transduction limits the amount of ethylene that is produced by a plant as a consequence of the addition of exogenous IAA. IAA plays a dual role in promoting cell proliferation and elongation while at the same time stimulating the transcription of ACC synthase, one of the key steps in ethylene synthesis. In this way, ethylene may limit the amount of its own synthesis. By lowering the amount of ethylene, ACC deaminase relieves the ethylene repression of auxin response factor synthesis. This results in an increase in the IAA stimulation of growth without causing an increase in ethylene production.

The observation that mutant strain *P. putida* UW4/AcdS⁻ did not promote root elongation as well as the wild type is consistent with previous results (Li et al. 2000). Although an ACC deaminase overexpressing *P. putida* UW4 strain was not constructed, a derivative of the endophytic plant-growth-promot-

ing bacterium *Burkholderia phytofirmans* PsJN that overexpressed ACC deaminase compared to the wild type had a somewhat higher level of plant root elongation activity than the wild-type (Y. Sun, Z. Cheng, and B. R. Glick, *unpublished data*).

The effect of canola root exudates on protein expression was similar for wild-type *P. putida* UW4 and mutant *P. putida* UW4/AcdS⁻ (Fig. 5), with the majority of identified significant protein expression changes occurring in both the wild type and the AcdS⁻ mutant. This is not unexpected because the *P. putida* UW4 ACC deaminase is unlikely to be involved in bacterial perception and response to plant host signals, and it causes a noticeable difference only in plant growth. Nevertheless, we can draw the conclusion that the presence or absence of ACC deaminase has no significant impact on detected *P. putida*

Fig. 3. A, Expression of the His-tagged recombinant proteins was confirmed by Western blotting. In the case of OmpF protein expression, two-dimensional electrophoresis was performed to separate the expressed His-tagged protein from a background band with similar molecular weight. **B**, Disruptions of target genes in the *Pseudomonas putida* UW4 genome were confirmed by diagnostic polymerase chain reaction (PCR) for individual genes. In each case, the PCR products from the mutant strains were 1.5 kbp longer than their alleles in the wild type, reflecting the insertion of the *tetA* gene into each of these genes.

Fig. 4. Root length of 7-day-old canola seedlings. Letters represent results of analysis of variance of seedling root length, where statistical significance is indicated by a different letter. Error bars represent standard error of the mean with 67 to 70 plants per treatment. The asterisk indicates there is no root length data available for the UW4/Pdf-treated plants because the Pdf protein disrupted strain was not viable.

Fig. 5. Plot of protein expression changes in wild-type *Pseudomonas putida* UW4 in the presence of plant root exudates versus changes in the mutant *P. putida* UW4/AcdS⁻. Overall, similar changes in expression were observed in both treatments.

UW4 gene expression on the exposure to canola root exudates; in other words, the wild-type *P. putida* UW4 and mutant strain UW4/AcdS⁻ respond similarly to host signals in root exudates. On the other hand, when ACC deaminase minus mutants of *B. phytofirmans* PsJN were examined, their physiology was significantly changed compared with the wild type, indicating changes in expression levels of at least some proteins (Y. Sun, Z. Cheng, and B. R. Glick, *unpublished data*). These observations prompted us to investigate the proteomic profile of *P. putida* UW4/AcdS⁻ along with the wild type. However, the differential in-gel electrophoresis (DIGE) analysis and especially the subsequent mass spectrometric analysis were both biased in that they largely examined highly abundant proteins. Thus, some less-abundant proteins that were differentially regulated in the wild-type *P. putida* UW4 and the AcdS⁻ mutant were not readily detectable on the two-dimensional (2-D) gels or below the sensitivity level of the mass spectrometer.

Many of the proteins that are upregulated in response to canola root exudates are involved in the utilization of nutrients (Fig. 2; Table 1). This observation is consistent with a transcriptomic study of bacterial responses to plant root exudates

(Mark et al. 2005) and with the notion that the utilization of the major components in plant root exudates by pseudomonads is the nutritional basis for their colonization of host plant roots (Lugtenberg et al. 1999). Another group of proteins whose expression is increased participate in bacterial cell envelope synthesis, including exopolysaccharide and peptidoglycan biosynthesis and membrane lipoprotein localization, which are thought to be essential for bacterial root colonization, symbiosis, virulence, or even viability (Dekkers et al. 1998; Gao et al. 2001; Tanaka et al. 2001; Yorgey et al. 2001). In addition, a few other upregulated proteins, such as GTP-binding protein TypA, chaperone clpB, and a ThiJ/PfpI family protein, were all previously shown to be required during the interaction of bacteria with their plant hosts (Caldelari et al. 2006; Hughes et al. 2007; Kiss et al. 2004).

On the other hand, a protein involved in chemotaxis as a two-component response regulator, CheY, was downregulated by canola root exudates (Fig. 2; Table 1). This finding is in agreement with a previous observation (Mark et al. 2005) and with the idea that chemotaxis plays important roles in bacterial host selection and colonization (Mark et al. 2005) and re-

Table 1. Differentially expressed proteins identified by tandem mass spectrometry^a

Spot, accession number	Protein	MW (kDa) ^b	pI ^b	Score ^c	SC ^d	WT ratio ^e	WT <i>P</i> value	AcdS ⁻ ratio ^e	AcdS ⁻ <i>P</i> value
Cell envelope synthesis									
1, Q4KJY3_PSEF5	dTDP-glucose synthase	32.6	5.33	81.9	16.49	1.5	0.0017	2.29	0.011
2, Q3K5U7_PSEPF	Eibulose-phosphate 3-epimerase	24.1	4.95	94.21	40.62	2.81	0.00002	3.04	0.0081
3, Q3K8P9_PSEPF	Ubiquinone oxidoreductase	60.8	5.65	99	10	2.31	0.00002	1.95	0.019
4, Q4K8M4_PSEF5	3-Semethylubiquinone-9 3-methyltransferase	26.0	5.69	84.01	26.29	1.07	0.44	4.28	0.0058
5, Q48P39_PSE14	RfaE bifunctional protein	50.2	5.54	87.68	10.13	8.96	0.00044	1.82	0.00089
6, Q4KJM6_PSEF5	Periplasmic glucan biosynthesis protein MdoG	67.8	6.86	61.41	5.25	1.97	0.00002	1.86	0.0039
7, Q3K746_PSEPF	D-alanine—D-alanine ligase	34.4	5.02	93.49	14.2	1.66	0.0013	1.74	0.05
8, Q3KA81_PSEPF	Outer membrane lipoprotein carrier LolA	20.4	5.11	90.65	26.21	1.59	0.00058	2.05	0.032
9, Q4KGQ4_PSEF5	Protease Do subfamily, peptidase MucD	50.7	6.20	64.45	11.55	1.64	0.029	1.77	0.04
Membrane transportation									
10, Q48FB5_PSE14	Porin D	47.0	4.94	60.66	4.32	3.5	0.00002	6.58	0.00009
11, Q9XL6_PSEFL	Outer membrane protein F	36.6	4.68	90.82	13.08	7.57	0.0015	7.09	0.0055
12, Q4KHG8_PSEF5	Outer membrane protein OMP85	87.4	5.21	78.1	12.69	5.38	0.00022	5.21	0.0046
13, Q9HT68_PSEAE	TonB-dependent receptor	28.1	5.79	47.86	10.77	1.52	0.00076	2.11	0.0036
14, Q3K503_PSEPF	Extracellular solute-binding protein, family 1	32.2	6.31	93.93	20.46	4.31	0.00096	7.85	0.00058
15, Q3KHP0_PSEPF	Extracellular solute-binding protein, family 3	34.1	5.54	98.37	22.45	6.79	0.00006	4.76	0.00084
16, Q3K7H7_PSEPF	Periplasmic substrate-binding protein	35.6	5.72	94	10	1.61	0.015	2.76	0.0018
17, Q3K5H9_PSEPF	ABC-type glycine betaine transport system	32.0	5.85	99.24	28.25	2.99	0.00002	4.1	0.028
18, Q4KH87_PSEF5	Amino acid ABC transporter	27.8	5.82	99	25.38	1.71	0.0032	3.33	0.0031
19, Q88NR4_PSEPK	Branched-chain amino acid ABC transporter	39.4	6.02	44.09	3.23	2.78	0.0013	3.41	0.00035
20, Q4KAH1_PSEF5	YhgI protein	21.0	4.57	36.66	6.7	2.36	0.0031	3.67	0.019
21, Q4KCT4_PSPF5	Nonspecific acid phosphatase	38.1	5.80	43.02	5.87	5.02	0.0013	10.91	0.00041
22, Q87TT2_PSESM	ATP synthase subunit alpha	55.3	5.46	100	38.33	9.32	0.011	1.53	0.0088
Nucleotide acid metabolism									
23, Q3KIY4_PSEPF	Adenylosuccinate sythase	46.5	5.57	99.92	33.1	1.7	0.0031	2.87	0.0033
24, Q4ZTK7_PSEU2	AP endonuclease, family 1	31.1	5.89	74.92	8.15	1.78	0.017	1.82	0.0021
Amino acid metabolism									
25, Q4KK38_PSEF5	ACC deaminase	35.2	5.37	84.15	9.06	1.64	0.0012	N/A	N/A
26, Q4KDP6_PSEF5	Branched-chain amino acid aminotransferase	36.9	6.19	69.37	6.78	1.7	0.043	1.73	0.017
27, Q3KJX4_PSEPF	4-Aminobutyrate aminotransferase	44.8	5.91	99.83	31.76	1.72	0.0092	2.41	0.031
28, Q4K8M8_PSPF5	Phosphoserine aminotransferase	40.0	5.04	41.82	2.75	3.21	0.002	5.02	0.0023
29, P80064_PSEUJ	4-Hydroxyphenylpyruvate dioxygenase	40.1	5.23	99.65	31.37	4.44	0.00013	7.52	0.009
30, Q4KJN8_PSEF5	Urocanate hydratase	61.3	5.79	74.19	6.61	1.82	0.0034	1.13	0.57
Carbohydrate metabolism									
31, Q3K5F5_PSESF	Fructose-bisphosphate aldolase	38.5	5.48	94.52	24.86	3.16	0.014	2.9	0.0022

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^a Accession numbers refer to the top database match. Expression ratios and *P* values were determined using differential in-gel electrophoresis analysis on triplicate bacterial cultures. Numbers in bold indicate proteins expression that was not significantly changed. Accession number of top database match from SwissProt *ExPASy* database (Gasteiger et al. 2003).

^b MW and pI were calculated from amino acid sequence of the top database match using the *ExPASy* Compute pI/MW tool (Gasteiger et al. 2003) and compared with gel location.

^c PEAKS match score.

^d PEAKS sequence coverage.

^e Wild type (WT) and mutant expression ratios relative to control, with upregulated proteins calculated as treated/control and downregulated proteins calculated as -(control/treated). N/A = not available.

sponse to environmental stimuli (Nixon et al. 1986). The expression of a transcriptional regulator, a TraR/DksA family protein involved in quorum sensing, was also downregulated by canola root exudates. Previous studies have shown that the quorum-sensing circuitry that is operative in *P. aeruginosa* is essential for the expression of its virulence factors as well as for its biofilm formation when colonizing lungs of cystic fibrosis patients (Singh et al. 2000). In addition, expression of an essential protein in bacterial protein synthesis (peptide deformylase), a Fis family protein involved in DNA repair or modification, and a previously uncharacterized protein were also decreased more than twofold by canola root exudates.

Although some *P. putida* UW4 proteins that facilitate the establishment of host-bacterial interactions were upregulated in response to canola root exudates, some essential proteins were downregulated, which should inhibit bacterial growth. This downregulation could reflect the fact that, in the presence of sufficient resources, the bacterium no longer needs to synthesize proteins to the same extent as in the absence of nutrients. Again, this complexity in changing protein expression level in response to root exudates would appear to reflect a

major shift in the physiology of bacteria that are associated with plant roots rather than free living.

The roles played by selected proteins that were differentially expressed in the presence of root exudates were investigated by overexpression or knockout of the corresponding genes. In the first instance, it was ascertained that the plant-growth-promoting activity of *P. putida* UW4 depends on the outer membrane protein OmpF. The OmpF porin is a trimetric integral membrane protein which forms a nonspecific transport channel responsible for passive transport of small molecules, such as nutrients and waste products, across the outer membrane of gram-negative bacteria (Cowan et al. 1995). The OmpF protein is also thought to be involved in adhesion and invasion of the Crohn's disease-associated *Escherichia coli* LF82 (Rohlfson et al. 2007). The fact that the OmpF protein overexpression and elimination in *P. putida* UW4 led to an increase and decrease, respectively, of its root elongation activity (Fig. 4) can be explained by two mechanisms. The plant-growth-promoting activity of *P. putida* UW4 relies on the transport of ACC from plant cells into attached bacterial cells, because ACC deaminase is localized and functions in the bacterial cytosol. Disrup-

Table 1. (continued from preceding page)

Spot, accession number	Protein	MW (kDa) ^b	pI ^b	Score ^c	SC ^d	WT ratio ^e	WT P value	AcdS ⁻ ratio ^e	AcdS ⁻ P value
Carbohydrate metabolism (Continued)									
32, O05137_PSEFL	Malate synthase G	78.9	5.66	89.33	5.1	1.57	0.0031	1.58	0.00008
33, Q116I3_9PSED	Malate dehydrogenase	60.1	5.89	73.68	4.22	1.86	0.00006	2.3	0.0041
34, Q3KF24_PSEPF	3-Isopropylmalate dehydratase	50.7	5.67	92.3	12.71	2.03	0.015	1.19	0.24
35, Q4K9U5_PSEF5	Isocitrate dehydrogenase	45.4	5.27	99.97	27.75	9.27	0.0034	9.04	0.0098
36, Q4K9P7_PSEF5	Isovaleryl-CoA dehydrogenase	42.4	5.74	60.43	6.46	1.69	0.0036	2.47	0.017
37, Q3KFE9_PSEPF	Isocitrate lyase and phosphorylmutase	32.0	5.46	95.24	20.95	1.28	0.057	3.37	0.0059
38, Q883Z4_PSESM	Succinyl-CoA synthase	41.2	5.70	98.07	28.35	3.3	0.0022	4.06	0.0003
39, Q4KKA2_PSEF5	Succinate-semialdehyde dehydrogenase	51.6	5.75	96.18	14.37	1.77	0.00077	2.23	0.0048
40, Q3K7K0_PSEPF	Argininosuccinate synthase	45.4	5.38	77.93	7.41	1.67	0.0092	2.62	0.0022
41, Q3K4C9_PSEPF	Oxaloacetate decarboxylase	65.4	5.60	80	3	1.84	0.012	1.56	0.021
42, Q3K7R9_PSEPF	Pyruvate kinase	52.1	6.04	97.22	15.53	6.13	0.00052	5.82	0.00053
43, Q3KC97_PSEPF	Acyl-CoA dehydrogenase	40.9	5.25	95.43	20.46	1.78	0.01	3.63	0.0038
Transcription regulation									
44, Q51455_PSEAE	Chemotactic responder CheY	13.9	6.10	33.02	19.35	-2.88	0.024	-2	0.00062
45, Q3K6E1_PSEPF	Periplasmic ligand-binding sensor protein	26.6	4.34	85.76	8.73	-2.83	0.00016	-1.63	0.029
46, Q3KI42_PSEPF	Transcriptional regulator, Fis family	20.5	5.13	91.3	30.65	-3.31	0.00006	-4	0.014
47, Q3K6R3_PSEPF	Transcriptional regulator TraR/DksA family	16.9	5.21	62.4	20.41	-2.38	0.00002	-2.64	0.023
48, Q3KDY8_PSEPF	NmrA-like protein	26.8	5.15	75.2	13.55	2.34	0.01	2.15	0.016
49, Q113U3_9PSED	Transcriptional regulator PyrR	18.7	5.01	39.15	9.88	-2.5	0.00038	-1.62	0.044
Protein synthesis, folding and degradation									
50, Q4K517_PSEF5	Tyrosyl-tRNA synthase	44.2	5.49	90.7	10.78	2.36	0.025	2.94	0.003
51, Q3K918_PSEPF	Elongation factor P/Yeip	21.0	4.72	99.43	36.51	-1.73	0.0023	-1.56	0.026
52, Q886P0_PSESM	Ribosome recycling factor	20.5	6.76	46.7	8.65	-2.37	0.015	-2.18	0.042
53, Q88PX7_PSEPF	Ribosomal 5S rRNA E-loop binding protein	21.1	5.72	92.2	10.14	-2.76	0.00001	-1.47	0.018
54, Q3KJG5_PSEPF	GTP-binding protein TypA	67.1	5.40	95.1	9.9	3.61	0.00004	3.67	0.0023
55, Q4K5T2_PSEF5	Peptidyl-prolyl cis-trans isomerase	21.7	4.69	93.87	32.68	-1.71	0.00002	-1.9	0.0012
56, Q4K4X7_PSEF5	Survival protein SurA	46.2	5.30	66.17	9.09	1.79	0.00005	1.67	0.033
57, Q3K6N3_PSEPF	Chaperone clpB	95.6	5.39	97.8	12.76	4.57	0.00005	4.04	0.00027
58, Q3KJB6_PSEPF	Heat shock protein HslU	50.0	5.76	92.5	9.89	4.43	0.0006	7.17	0.013
59, Q4KIH2_PSEF5	Co-chaperone GrpE	20.8	4.65	92.2	35.64	-1.95	0.0012	-2.26	0.0045
60, A5W7L4_PSEPU	Carboxyl-terminal protease	75.0	6.10	95	3	1.87	0.00014	2.03	0.009
61, Q3KKE5_PSEPF	Peptide deformylase	19.4	5.17	82.91	23.81	-6.94	0.00035	-6.45	0.016
62, Q4KGZ6_PSEF5	ThiJ/PtpI family protein	20.7	5.57	46.41	6.74	4.03	0.00017	4.93	0.00007
Oxidoreductase									
63, Q3K4N3_PSEPF	Aldehyde dehydrogenase	53.2	5.37	96.81	13.88	1.62	0.0066	5.18	0.011
64, Q48FA2_PSE14	Ferredoxin-NADP reductase	29.7	5.32	97.54	29.73	3.23	0.0034	2.2	0.0031
65, Q3KE62_PSEPF	Peroxidase/catalase	82.7	5.30	75.62	4.37	7.38	0.00004	6.75	0.026
66, Q4ZY90_PSEU2	Thiolase	40.4	5.91	89.57	6.89	-1.51	0.0053	-2.07	0.09
67, Q4KH19_PSEF5	Xenobiotic reductase B	37.4	5.53	96.43	16.33	-2.53	0.00003	-1.81	0.036
68, Q3K7J6_PSEPF	Alkyl hydroperoxide reductase	21.8	5.08	97.53	41	-3.33	0.00008	-3.92	0.004
69, Q3KGGH2_PSEPF	Nitroreductase	22.0	5.79	85.9	20.3	-2.32	0.0033	-2.31	0.065
Others									
70, Q4KFS1_PSEF5	Uncharacterized protein	19.4	4.48	90.24	32.57	-3.02	0.0073	-2.1	0.049
71, Q3K9Y9_PSEPF	Hypothetical protein A	25.1	4.94	77.45	23.93	1.56	0.0028	2.29	0.11
72, Q4ZNZ4_PSEU2	Cell division protein FtsA	44.6	5.26	91.12	11.24	1.5	0.0026	1.75	0.018

tion or enrichment of the OmpF protein, which potentially transports ACC through the *P. putida* UW4 outer membrane, may directly affect its plant-growth-promoting ability. In this regard, changes to the expression of the OmpF protein are predicted to have little or no impact on the growth-promoting activity of the ACC deaminase minus mutant of strain UW4. Alternatively, the plant-growth-promoting activity of *P. putida* UW4, which is dependent on the direct physical binding of bacterial cells to plant surfaces, may also be dependent on the OmpF protein that may be involved in bacterial cell adhesion to the plant.

Second, based on the expression results, canola root exudates appear to contain a bacterial peptide deformylase inhibitory compound, which could potentially be employed as an antibacterial agent candidate. Peptide deformylase (Pdf) is involved in protein maturation after translation through its activity to remove the *N*-formyl group of *N*-formyl methionine. The Pdf protein is essential in eubacteria and absent in mammalian cells, making it an appealing target for the selection of novel antibiotics (Leeds and Dean 2006). The fact that the Pdf-protein-disrupted strain was not viable suggests that this protein is essential in bacterial cells (Fig. 4). Downregulation of the peptide deformylase in response to plant exudates (Fig. 2; Table 1) may reflect a plant mechanism to inhibit bacterial growth and limit colonization by bacterial pathogens. The strain overexpressing the Pdf protein promoted root length to a greater extent than the wild type, possibly by overcoming the inhibitory effects of a putative plant compound. Previously, exudates containing plant-derived antibacterial metabolites were shown to confer tissue-specific resistance to various bacterial pathogens (Bais et al. 2005). For example, actinonin, a naturally occurring bacterially encoded antibacterial agent, was previously identified as a Pdf protein inhibitor (Chen et al. 2000). Thus, the presence of a bacterial peptide deformylase inhibitory compound in root exudates may provide an opportunity to derive a potent and broad-spectrum antibacterial agent.

The previously uncharacterized protein (Hyp) has been predicted to be a potential DNA binding protein (Marchler-Bauer et al. 2007). The observation that the Hyp protein may be involved in plant growth promotion has not been made previously. However, whether the Hyp protein is a DNA-binding protein possibly involved in gene transcriptional regulation is still conjecture at this point. Overexpression of the Hyp protein led to a growth inhibition of canola roots greater than that in the absence of bacteria (Fig. 4); therefore, it is possible that the Hyp protein has a role in regulation of genes related to virulence in *P. putida* UW4, causing detrimental effects on plant growth. Thus, the Hyp-protein-overexpressing strain not only abolished the plant-growth-promoting ability of *P. putida* UW4 but also may have transformed this bacterium into a deleterious organism that caused a decrease in root length compared with control plants (Fig. 4). Conversely, the Hyp protein knockout strain demonstrated an enhanced plant-growth-promoting ability, probably due to elimination of deleterious effects that could have been attributed to the Hyp protein. Again, downregulation of the expression of the Hyp protein in response to plant root exudates (Fig. 2; Table 1) may be another plant self-protection strategy.

Previous workers have investigated some of the interactions between plant-growth-promoting bacteria and plants using other strategies based on transcriptomic characterization (Ramos-Gonzalez et al. 2005; Verhagen et al. 2004; Wang et al. 2005). Here, proteomic profiling of the plant-growth-promoting bacterium *P. putida* UW4 in response to canola root exudates, followed by functional analyses, have suggested how some proteins may be involved in host–bacterial interactions. This

approach should contribute to a better understanding of how both beneficial and pathogenic bacterial strains establish interactions with their hosts, because many of the proteins identified in this work were previously shown to be involved in pathogenic bacterial or fungal infections of plants and animals (Caldelari et al. 2006; Dumas-Gaudot et al. 2004; Hughes et al. 2007; Yorgey et al. 2001). Moreover, this work provides strategies to both identify potential antibacterial agents and develop bacterial strains that might be of significance to agricultural or environmental clean-up strategies.

MATERIALS AND METHODS

Bacterial strains and growth.

The microbial strains and plasmids used in this study are described in Supplementary Table 1. *E. coli* DH5 α (Hanahan 1983) was used as the host strain for the construction and maintenance of recombinant plasmids. *E. coli* strains were cultivated aerobically in Luria-Bertani broth or plates (Fisher Scientific Co., Ottawa, ON, Canada) at 37°C. *P. putida* UW4 (Glick et al. 1995) and its mutant strains were cultivated aerobically in tryptic soy broth (TSB) or plates (Fisher Scientific Co.) at 30°C. If required, the medium was supplemented with antibiotics at final concentrations of ampicillin at 100 μ g/ml, kanamycin at 30 μ g/ml, or tetracycline at 15 μ g/ml.

Plant growth.

Plant growth followed a previously described procedure with minor modifications (Penrose and Glick 2003). Briefly, seed of *Brassica napus* var. Westar were surface sterilized with 70% ethanol. Then, the sterilized seed were treated at room temperature for 1 h with induced (for either ACC deaminase activity or the proteins listed in Figure 3A) bacterial suspensions, diluted with sterile 0.03 M MgSO₄ from an optical density at 600 nm (OD₆₀₀) of approximately 2 to a final OD₆₀₀ of 0.15. Sterile 0.03 M MgSO₄ solution-treated seed were used as a negative control. The treated and control seed (six per pouch) were planted in growth pouches (Cyg seed germination pouch; Mega International, St. Paul, MN, U.S.A.) containing 30 ml of sterile Milli-Q water. The growth conditions, including time period, light intensity, and temperature, were all performed as exactly as described (Penrose and Glick 2003). For canola root exudates collection, the remaining water in all growth pouches was pooled and filtered through 0.2- μ m nitrocellulose filters, and the filtrates were stored at –20°C in the dark. For plant-growth-promotion assays, plant root length was measured following 7 days of growth at 20°C in a growth chamber with a light intensity of 200 μ E and a day-and-night regime of 12 h of light.

Quantification of plant exudates components.

The quantity of ACC in plant exudates were measured by the Waters AccQ-Tag Amino Acid Analysis Method (Waters Chromatography Inc., Mississauga, ON, Canada) The derivatization, separation, and detection processes were done by following the Waters AccQ-Fluor user guide. Amino acid derivatives (including ACC) were separated by a Hewlett Packard high-performance liquid chromatography (HPLC) system (1050 Series Quaternary Pump equipped with a high-efficiency 4 m Nova-Pak C18 column), and then detected by a Hewlett Packard 104a Programmable Fluorescence Detector at an excitation wavelength of 250 nm and an emission wavelength of 395 nm. The flow rate was 1 ml/min and the gradient used was as described previously (Penrose and Glick 2001). The quantification of IAA was performed as described previously (Patten and Glick 2002), with modifications. Plant exudates were analyzed using a Perkin Elmer Series 4 HPLC equipped with an Allure

reverse-phase C18 column (5 μ m; 4.6 by 150 mm) (Restek Inc., Rockville, ON, Canada). The elution buffer was acetonitrile and 50 mM KH_2PO_4 (pH 3) (30/70) at a flow rate of 0.6 ml/min and the eluates were detected by a Micromeritics 788 Dual Variable detector at 220 nm.

Proteomic profiling and DIGE analysis.

Wild-type *P. putida* UW4 and mutant strain *P. putida* UW4/ ΔcdS^- were grown in 50 ml of 2 $\times$ DF minimal salts medium (Penrose and Glick 2003) supplemented with 50 ml of collected plant root exudates or 50 ml of sterile Milli-Q water to late-log phase for DIGE analysis.

The DIGE and the mass spectrometric analyses were done as follows. Briefly, proteins were extracted from bacterial cells by rotating the cells in ice-cold lysis buffer (2 M thiourea, 7 M urea, 4% CHAPS, 30 mM Tris, pH 8.5; 5 ml/g of wet cells) containing 100 μ l of protease inhibitor cocktail (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) for 30 min. The lysed cells were centrifuged at $150,000 \times g$ at 4°C for 90 min and the supernatant was saved at -70°C as the whole cell extract. Protein samples extracted from differently treated bacterial cells were labeled with different Cy5 dyes (GE Healthcare, Mississauga, ON, Canada) and, subsequently, mixed together and separated on the same 2-D gel. All bacterial cultures and analytical gels used in DIGE analysis were done in triplicate. After 2-D electrophoresis, matching and statistical analyses of all protein spots were performed using the DeCyder V 6.0 software (GE Healthcare, Mississauga, ON, Canada). Proteins with a significant change in expression level were excised from preparative 2-D gels stained overnight with Bio-Safe Coomassie (Bio-Rad Laboratories, Mississauga, ON, Canada). The gel pieces containing proteins of interest were washed, destained, reduced, alkalized, dried, and then digested overnight with trypsin (Promega Corp., Madison, WI, U.S.A.). The tryptic peptides were recovered and cleaned using the C-18 ZipTip system (Millipore, Billerica, MA, U.S.A.) and eluted with 5 μ l of 50% acetonitrile containing 0.2% formic acid. The protonated peptides were analyzed using a Waters Micromass Q-TOF Ultima with nanospray injection as the sample delivery method. Proteins were identified using PEAKS software (v. 3.1; Bioinformatics Solutions Inc., Waterloo, ON, Canada) (Ma et al. 2003) against sequences in the Swiss-Prot database (Gasteiger et al. 2003).

Expression and gene knockout in *P. putida* UW4.

The *Pseudomonas*-*Escherichia* shuttle expression vector pETP was constructed by inserting the origin of replication (ori1600) from pUCP26 (West et al. 1994) into the *Sph*I site of the expression vector pET30a(+) (Novagen, Hornby, ON, Canada). Using primers with either the original (PO) or the incorporated (PI) *Sph*I site, the 1,557-bp fragment containing ori1600 was amplified from pUCP26 by PCR. Both the PCR fragment and the backbone vector, pET30a(+), were digested with *Sph*I and the fragment was inserted into pET30a(+). The resulting plasmid was designated as pETP.

The genes encoding the four selected proteins (Fig. 3A) were amplified by PCR using primers designed based on conserved up- and downstream regions in other *Pseudomonas* genomes (*P. fluorescence* strains Pf-5 and PfO-1 and *P. putida* strains KT2440 and F1). The PCR products were cloned into pGEM-T easy (Promega Corp.). The DNA sequences of these four genes were determined at the Biology Molecular Sequencing Facility, York University (Toronto). Four coding regions were cloned into pGEM-T easy using four sets of primers. The inserts, released by *Not*I digestion, were subcloned into the *Not*I site of pETP for expression in *P. putida* UW4. Plasmid constructs were resequenced to ensure that genes were inserted

in the right reading frame and that no alternations to the DNA sequence had taken place. Plasmids were introduced into *P. putida* UW4 by electroporation as described (Smith and Iglewski 1989). The expression of the 6 $\times$ His-tagged recombinant proteins was confirmed by Western blotting before these strains were used to treat canola seed in the root elongation growth pouch assay. 2-D Western was performed to separate the expressed OmpF protein from a large background band with the same molecular weight in the control sample (*data not shown*). The 6 $\times$ His-tagged proteins were detected using a mouse monoclonal His-tag antibody (Novagen, Hornby, ON, Canada), and an HRP-conjugated anti-mouse immunoglobulin G secondary antibody (Sigma-Aldrich Canada Ltd.). The immune complexes were visualized by chemiluminescence using an ECL Plus Western blotting detection kit (GE Healthcare).

The mutant strains *P. putida* UW4/ Δfis^- , UW4/ Δhyp^- , and UW4/ ΔompF^- were constructed by disrupting the *fis*, *hyp*, and *ompF* genes, respectively. A tetracycline resistance gene was inserted within the coding region of each of these genes by homologous recombination as described previously (Li et al. 2000). For example, the pBR322 *Eco*RI-*Eco*88I fragment containing the tetracycline resistance gene (*tetA*) (Peden 1983) was inserted into the *Msc*I site within the *fis* coding sequence to make pGEM-Fis-tet. The pGEM-Fis-tet *Not*I fragment was then subcloned into the *Sma*I site of the pK19mobsacB, which facilitates gene disruption and allelic exchange by homologous recombination (Schafer et al. 1994). The resulting plasmid pRep-Fis was transformed into *P. putida* UW4 by electroporation (Smith and Iglewski 1989) and the transformants were selected on TSB plates containing tetracycline and sucrose. All mutant strains were tested for the disruption of the target genes by diagnostic PCR.

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