Expression of the *aggA* Locus of *Pseudomonas putida* *In Vitro* and *In Planta* as Detected by the Reporter Gene, *xylE*

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***In vitro* agglutinability by *Pseudomonas putida*, isolate Corvallis, with a plant root surface agglutinin is correlated with rapid adhesion of cells of the fluorescent pseudomonad to bean (*Phaseolus vulgaris*) root surfaces. Agglutinability in *P. putida* cells is regulated by nutrient status as well as growth phase. Cells grown in three different nutrient complex media are agglutinable at early and mid-late logarithmic phase but become nonagglutinable at stationary phase. Cells grown in a minimal medium are weakly agglutinable, but the addition of lysine, aspartic acid, or histidine increases agglutinability. Cells in the same minimal medium supplemented with bean root surface components grow in a highly agglutinated state. Previous data indicate both agglutination and rapid adhesion to roots by *P. putida* Corvallis involves the *aggA* locus, which contains two putative open reading frames (ORF), ORFAGG1 and ORFAGG2, on complementary strands. Sequence and deletion analyses suggest ORFAGG1 is the most probable ORF responsible for agglutination and adhesion. Chimeric fusion of an *Escherichia coli lac* promoter with ORFAGG1, but not with ORFAGG2, complemented agglutinability of an *aggA*::Tn*5* *P. putida* Agg$^-$ mutant, providing further evidence that ORFAGG1, not ORFAGG2, is responsible for agglutination. Heterologous expression of ORFAGG1 yields a 50-kDa precursor and a 48-kDa mature periplasmic protein. Fusions of ORFAGG1 and ORFAGG2 to the reporter gene, *xylE*, and detection of the reporter enzyme, catechol-2,3-oxygenase reveal an active promoter in the 5′ noncoding region of ORFAGG1. The ORFAGG1 promoter is active during growth of the cells in liquid culture and is regulated by growth medium. Greatest activity of the catechol-2,3-oxygenase is observed in stationary phase when the cells are nonagglutinable. Expression of the ORFAGG1 promoter is detected in *P. putida* cells extracted from the root surface of bean at 48 and 72 hr after inoculation.**

Certain isolates of *Pseudomonas putida* and *P. fluorescens* have biocontrol and/or plant growth promotion potential (Weller 1988). These effects are attributed to specific products from the pseudomonads including siderophores, 2,4-diacetylphloroglucinol, hydrogen cyanide, phenazines, and plant-growth promoting compounds (Bakker *et al.* 1986; Hamdan *et al.* 1991; Keel *et al.* 1992; Thomashow and Weller 1988; Thomashow *et al.* 1990; Voisard *et al.* 1989; Weller 1988). In addition to direct detection of compounds from the rhizosphere (Keel *et al.* 1992; Thomashow *et al.* 1990), the role of these bacteria has been supported by other genetic tagging of a locus (loci) and comparison of the ability of wild type, mutant, and complemented mutant to provide biocontrol and/or plant-growth promotion.

Effective populations of the beneficial bacteria on the root surface are essential in establishing biocontrol (Bull *et al.* 1991; Scher *et al.* 1985; Weller 1988). We have examined genes in a beneficial strain of *P. putida* which are involved in its ability to colonize plant roots. Agglutinability of *P. putida* isolate Corvallis by plant root glycoproteins has a critical role in rapid attachment of the bacterial cells to the root surface and in colonization of the rhizoplane (Anderson *et al.* 1988; Tari and Anderson 1988). The *aggA* locus of *P. putida* Corvallis is involved in both agglutination and adhesion to root surfaces (Buell and Anderson 1992). Sequence analysis of the *aggA* locus indicated two open reading frames (ORFAGG1 and ORFAGG2) were present on complementary strands of a 2.7-kbp *Eco*RI-*Hin*dIII fragment from *P. putida* (Buell and Anderson 1992). We proposed that ORFAGG1 was the most probable reading frame responsible for the agglutination phenotype because promoter and ribosome binding site consensus sequences were present. ORFAGG1 was predicted to encode a 50-kDa protein with a potential signal peptide cleavage site at amino acid residue 22, suggestive that the protein can be processed out of the cytoplasm. Although ORFAGG2 was predicted to encode a 43-kDa protein, it was a less probable candidate as it lacks ribosome-binding and promoter consensus sequences and has a putative GTG translational start codon (Buell and Anderson 1992). In this paper, we provide further evidence through chimeric fusions and heterologous expression studies that ORFAGG1, not ORFAGG2, is the reading frame involved in the agglutination phenotype at the *aggA* locus.

Regulation of expression of genes involved in biocontrol by other pseudomonads is controlled by nutrient availability. Gutterson (1988) demonstrated glucose to regulate at the transcriptional level antibiotic biosynthesis in *P. fluorescens* HV37a. Loper and Lindow (1991) demonstrated expression of a pyoverdine biosynthetic gene in

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P. fluorescens was regulated by iron deficiency. In addition, expression of the *hrp* loci, which govern hypersensitivity and pathogenicity in pathogenic *Pseudomonas, Xanthomonas,* and *Erwinia* species, is modulated by addition of carbon and nitrogen sources to minimal medium (Schulte and Bonas 1992; Willis *et al.* 1991). Expression of the *avrB* gene in *P. syringae* pv. *glycinea,* which confers cultivar specificity, is also regulated *in vitro* by specific carbon sources (Huynh *et al.* 1989). Consequently, we used the reporter gene, *xylE,* which encodes catechol-2,3-oxygenase (C23O; Gibson 1971; Walter *et al.* 1987), to identify potential promoter regions of ORFAGG1 and ORFAGG2. We studied the *in vitro* regulation of *aggA* promoter-*xylE* fusions under various nutrient and growth conditions. *In planta* expression of the *aggA* promoter-*xylE* fusion was studied to provide direct evidence of *aggA* expression in *P. putida* cells on plant root surfaces.

RESULTS

Hybrid p*lac-aggA* fusions.

Construction of the plasmid p*lac*-ORFAGG1 allowed identification of the ORF in *aggA* responsible for complementation of agglutination in *P. putida* Agg$^-$ mutant 5123 (Fig. 1). The plasmid, p*lac*-ORFAGG1, with a promoterless ORFAGG1 downstream from the *lac* promoter, complemented the Agg$^-$ mutant 5123 to wild-type levels of agglutinability, whereas p*lac*-ORFAGG2 (Fig. 1), with ORFAGG1 on the complementary strand did not complement agglutination in mutant 5123.

Heterologous expression of *aggA*-encoded proteins.

The generation of heterologous transcriptional fusions between a T7 promoter and ORFAGG1 allowed detection of ORFAGG1-encoded protein products in *E. coli* cells. Expression of the pT7-ORFAGG1 fusion in BL21(DE3) expression cells yielded two novel protein bands of 50 and 48 kDa, which were not detected when the vector alone was used (Fig. 2). The ORFAGG1-encoded 48-kDa band was detected in both total and periplasmic protein preparations, whereas the 50-kDa band was observed only in the total protein preparation (Fig. 2). Pulse-chase experiments indicated a 50-kDa protein was processed into a 48-kDa protein (data not shown). The pT7-ORFAGG2 fusion construct did not direct any novel protein synthesis in the *E. coli* expression system (Fig. 2).

Selection of optimal *aggA* promoter-*xylE* constructs.

C23O activity was determined in transconjugant *P. putida* cells grown to mid-late logarithmic phase in rich me-

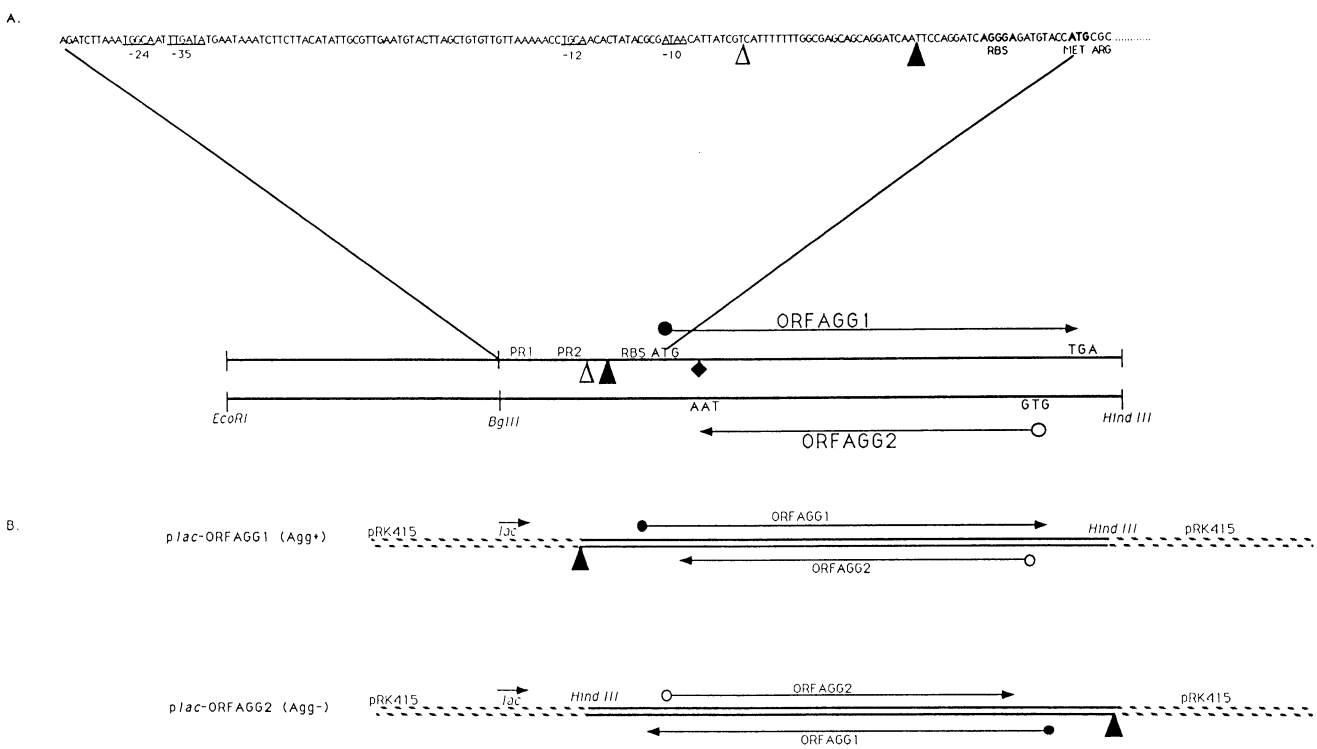

Fig. 1. The *aggA* locus. **A,** Salient restriction enzyme sites, coding, and noncoding regions of ORFAGG1 and ORFAGG2 are indicated. The 5′ noncoding sequence of ORFAGG1 is shown above. RBS = ribosome binding site; PR1= −24 and −35 promoter consensus sequences; PR2 = −12 and −promoter consensus sequences; ATG, GTG = translational start codons; TGA, TAA = translational stop codons; ▲ = terminus of *aggA* deletion clones p*lac*-ORFAGG1 and p*lac*-ORFAGG2; △ = terminus of *aggA* deletion clones pORFAGG1-951 and pORFAGG1-105; ◆ = terminus of *aggA* deletion clones pORFAGG1-1040 and pORFAGG1-188. **B,** Chimeric p*lac-aggA* fusions. The *aggA* deletion clones, p*lac*-ORFAGG1 and p*lac*-ORFAGG2, both contain a 1,664-bp fragment of the *aggA* locus. p*lac*-ORFAGG1 has a promoterless ORFAGG1 (1,356 nucleotides) positioned immediately downstream of the *lac* promoter in the pRK415 vector and is able to complement *P. putida* Agg$^-$ mutant 5123 to wild-type levels of agglutinability. p*lac*-ORFAGG2 has ORFAGG2 (1,254 nucleotides) positioned downstream of the pRK415 *lac* promoter with a promoterless ORFAGG1 located on the complementary strand and is unable to complement agglutination in the Agg$^-$ mutant 5123. The dark line represents *aggA* and the striped line represents the vector pRK415. The figure is not drawn to scale.

dium possessing fusions of *xylE* to either ORFAGG1 or ORFAGG2. Transconjugant *P. putida* cells containing the pRK415-*xylE* fusion had only trace C23O activity (data not shown). Only trace C23O activity was detected in transconjugants containing the promoterless ORFAGG1-*xylE* construct (pORFAGG1-751), with only 751 bp of 5′ noncoding region, or the fusion with all 2.7 kbp of the ORFAGG1 strand of the *aggA* locus (pORFAGG1-2646) (Fig. 3). Higher C23O activity was detected in nine other transconjugants, especially in the transconjugant bearing pORFAGG1-951, which contained a fusion of *xylE* most proximal to the predicted promoter in ORFAGG1. High C23O activity was also detected in *P. putida* transconjugants containing 188- and 105-bp segments of the putative ORF1 promoter, pORFAGG1-188 (10–21×) and pORFAGG1-105 (6–12×) respectively.

Fusions to *xylE* with the ORFAGG2 strand of the *aggA* locus yielded *P. putida* transconjugants with minimal to no C23O activity (Fig. 3). The construct, pORFAGG2-849, yielded the most activity with 15 nmoles product/min/mg of protein detected in the mid-late logarithmic phase culture. Additional analyses of active promoter regions of each ORF was limited to pORFAGG1-951 for the ORFAGG1 promoter and pORFAGG2-849 for ORFAGG2. *P. putida* (pRK415-*xylE*) transconjugants were used as a control.

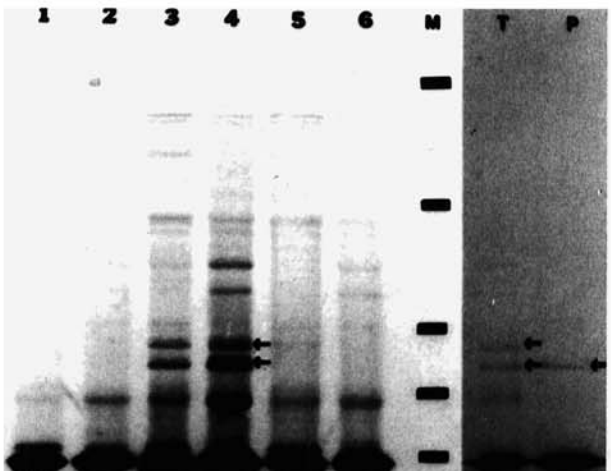

Fig. 2. Fluorogram of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of ^{35}S-methionine-labeled proteins from *E. coli* BL21(DE3) expression cells. Fusions of ORFAGG1 and ORFAGG2 to the 77 promoter of pET11 were made as described in the text. *E. coli* expression cells were labeled with ^{35}S-methionine after induction with IPTG as described in the text. Total and periplasmic proteins were separated on a 7.5% SDS polyacrylamide gel prior to fluorography. Total protein preparations: Lane 1: pET11 − IPTG; lane 2: pET11 + IPTG; lane 3: pT7-ORFAGG1 − IPTG; lane 4: pT7-ORFAGG1 + IPTG; lane 5: pT7-ORFAGG2 − IPTG; lane 6: pT7-ORFAGG2 + IPTG. M = Rainbow protein molecular weight markers (Amersham, Arlington Heights, IL) are myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa). T = pT7-ORFAGG1 + IPTG total protein preparation. P = pT7-ORFAGG1 + IPTG periplasmic protein preparation. The positions of the 50- and 48-kDa ORFAGG1 encoded proteins are noted with an arrow.

Agglutination and C23O activity *in vitro*.

The relationship between expression of the *aggA* locus and agglutinability of cultured cells was studied. Agglutinability of *P. putida* transconjugant cells varied with growth phase and nutrient supply (Table 1, Figs. 4 and 5). Like wild-type parental cells, the transconjugant cells grown in nutrient complex media (rich, LB, or KB) were agglutinable throughout the logarithmic phase, but were not agglutinable in stationary phase (data not shown).

P. putida (pORFAGG1-951) transconjugant cells were nonagglutinable when grown in minimal medium (Fig. 5). Addition of bean root surface components to this minimal medium caused the cells to grow in an agglutinated form and to remain self-agglutinable upon assay with additional agglutinin *in vitro* (Fig. 5, data not shown). The addition of the root surface components resulted in a twofold greater cell density after 12-hr growth in comparison to cells grown in nonamended medium. Increased agglutinability was observed with addition of aspartic acid, histidine, and lysine to the minimal medium (Table 1). Other amino acids and carbon sources had no effects on agglutinability although they stimulated growth.

Expression from the *aggA* locus promoter in the pORFAGG1-951 transconjugant was also regulated by growth medium and age of the cells but not always in a manner related to expression of the agglutination phenotype. Expression, as measured by specific activity, was similar in Agg$^+$ cells grown in each of the three enriched media (rich-819 nmoles/min/mg, LB-820 nmoles/min/mg or KB-920 nmoles/min/mg; Fig. 4) and this level was higher than from Agg$^-$ cells grown in minimal media (BMM 389 nmoles/min/mg (Table 1); minimal 361 nmoles/min/mg; Fig. 5). However, C23O activity was markedly higher in stationary-phase cells (Fig. 5) that were not agglutinable. Similarly, amendments to minimal medium that enhanced agglutinability did not always increase C23O activity (Table 1). Slightly increased levels of C23O activity were observed consistently in amendments with glycine and pyruvate and reduced levels with methionine and mannitol (Table 1).

C23O activity *in planta*.

Very low C23O activity was detected in extracts of cells of *P. putida* (pORFAGG2-849) or *P. putida* (pRK415-*xylE*) harvested from the bean rhizosphere 48 and 72 hr after inoculation. Appreciable C23O activity was detected when an inoculum of *P. putida* (pORFAGG1-951) was used (Table 2). Although this inoculum possessed C23O activity, only trace C23O activity was detected in the processed extracts of cells harvested from the roots immediately after inoculation (Table 2). Cells recovered from roots after 48 and 72 hr of inoculation possessed high C23O-specific activities (Table 2), which were at levels similar to those expressed during growth on rich media (Fig. 5).

DISCUSSION

The *aggA* locus of *P. putida* is essential for expression of agglutinability with a plant agglutinin. The agglutination phenotype is involved in rapid attachment of cells to bean root surfaces (Buell and Anderson 1992). Previous analysis

of the *aggA* locus revealed the presence of two potential ORFs, ORFAGG1 and ORFAGG2, on complementary strands (Buell and Anderson 1992). Analysis of the *aggA* nucleic acid sequence suggests that ORFAGG1, not ORFAGG2, was the most probable ORF involved in agglutination. In this study, fusion analysis using a *lac* promoter from *E. coli* provides further evidence that ORFAGG1, and not ORFAGG2, is involved in generation of the agglutination phenotype. Only fusion of the *E. coli lac* promoter to the promoterless ORFAGG1 but not ORFAGG2 complemented agglutination in Agg$^-$ mutant 5123. In addition, only trace promoter activity was detected in fusions of the *xylE* reporter with ORFAGG2 and this cryptic activity was not localized in the regions 5′ to a potential GTG translational start codon. Consequently, we suggest that ORFAGG2 lacks a functional promoter region in 5′ regions of the predicted ORFAGG2 GTG translational start codon (Buell and Anderson 1992).

Expression of ORFAGG1 in an *E. coli* expression system suggests that ORFAGG1 encodes a 50-kDa protein which is processed into a 48-kDa periplasmic protein. This finding is consistent with the deduced amino acid sequence of ORFAGG1 containing a 22 amino-acid leader sequence preceding a potential 48-kDa protein. Localization of the processed AGGA protein in the periplasm of cells may reflect function of the protein in either generation or stabilization of the agglutination phenotype. Other rhizosphere bacteria have been demonstrated to have periplasmic components essential for attachment of the bacterial cells to plant structures. The periplasmic β-glucans in both *Rhizobium melilotii* and *Agrobacterium tumefaciens* are critical for successful attachment of the cells to plant surfaces (Smit *et al.* 1989; Dylan *et al.* 1990a, 1990b). Other attachment factors, flagella, pili, cellulose fibrils, and the adhesive protein, rhicadhesin (Winans 1992), also are processed via the periplasm. Detection of the AGGA protein in the periplasm is consistent with our findings (Buell and Anderson 1993) that Agg$^-$ mutants are altered in their periplasmic protein profiles, alterations which revert to wild-type profile upon complementation of the agglutination phenotype.

Characterization of the ORFAGG1 promoter region with the reporter gene, *xylE* found C23O activity to be maximal with a 951-bp fusion which placed the *xylE* reporter most proximal to the predicted ORFAGG1 promoter region. Fusion with 751 bp of the 5′ noncoding region of ORFAGG1 to *xylE*, a construct with the predicted promoter region deleted, yielded no reporter activity. Two

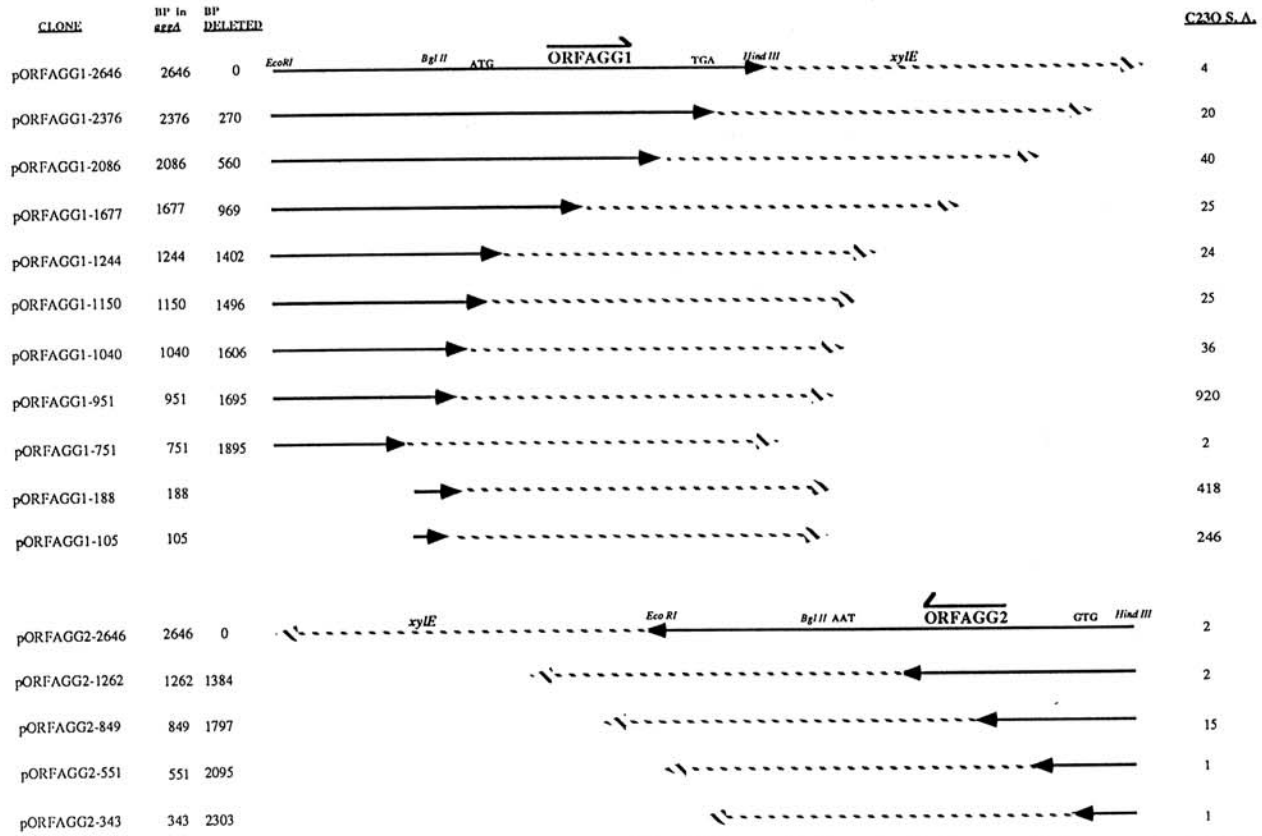

Fig. 3. *aggA-xylE* fusion constructs. Fusions of the ORFAGG1 or the ORFAGG2 strand of *aggA* with the promoterless reporter gene *xylE* were made as described in the text. The remaining base pairs of *aggA* and the number of base pairs deleted from the original 2.7-kbp of the *aggA* locus are reported. C23O activity, reported in nmol/min/mg of protein, is from agglutinable cells grown to mid-late logarithmic phase in rich medium as described in the text. All ORFAGG1-*xylE* fusions with the exception of pORFAGG1-951, pORFAGG1-751, and pORFAGG1-105, retain all or portions of the ORFAGG1 coding sequence fused 5′ of the *xylE* reporter gene. ORFAGG1 or ORFAGG2-*xylE* fusions are cloned in pRK415 such that all expression of *xylE* is independent of the pRK415 *lac* promoter. The dark line represents *aggA* and the striped line represents *xylE*. The figure is drawn to scale.

fusions, pORFAGG1-188 and pORFAGG2-105, which contain the predicted promoter consensus sequences (Buell and Anderson 1992) were also active. Detection of C23O activity from fusions with just a 105-bp 5′ noncoding region indicates no cryptic promoter activity was present in internal regions of ORFAGG1. Reduction in C23O expression in constructs with longer regions of the ORFAGG1 reading frame suggests that subtle effects on expression, perhaps due to three-dimensional structure, occur.

Agglutination of *P. putida* is regulated *in vitro* by both nutrient supply and growth phase. Rich nutrition enhances agglutinability, and the addition of root surface components to minimal medium causes agglutination. These findings suggest to us that the agglutination phenotype should be expressed in the rhizosphere, where the bacterial cells would encounter amino acids present in the root exudates. The observed expression from the ORFAGG1 promoter detected from cells in the bean rhizosphere suggests that the locus is transcribed in cells associated with the root surface. Consequently, this evidence strengthens the possibility that the agglutination phenotype is important in early colonization events of pseudomonads at root surfaces. However, promoter activity and expression of the agglutination phenotype are not well correlated in *in vitro* studies. Supplementation of minimal medium by root components did not consistently result in higher C23O activity, although the cells became agglutinated. Individual amino acid supplements also did not result in enhanced C23O activities even though agglutinability was enhanced. The major digression in the expression of C23O activity and agglutinability is observed in stationary phase. In stationary-phase cells grown on rich medium, the C23O activity is highly expressed, whereas the cells are nonagglutinable.

Table 1. The specific activity of C23O in extracts from transconjugant *Pseudomonas putida* (pORFAGG1-951) grown in BMM medium supplemented with various carbon and nitrogen compounds

Medium[a]	% C23O Activity[b]	Growth OD_{570nm}	Agg[c]
BMM	100 ± 0	0.33 ± 0.06	−
+ Root wash	96 ± 52	0.58 ± 0.10	3+
+ Aspartic acid	159 ± 98	0.86 ± 0.20	1+
+ Lysine	108 ± 48	0.41 ± 0.04	1+
+ Histidine	79 ± 31	0.81 ± 0.28	3+
+ Methionine	63 ± 9	0.35 ± 0.02	−
+ Glycine	157 ± 42	0.38 ± 0.02	−
+ Pyruvate	149 ± 39	0.57 ± 0.14	−
+ Citrate	99 ± 22	0.63 ± 0.14	−
+ Mannitol	64 ± 14	0.33 ± 0.03	−
+ Galactose	104 ± 33	0.30 ± 0.03	−
+ Arabinose	93 ± 17	0.31 ± 0.02	−
+ Fructose	161 ± 91	0.48 ± 0.08	−
+ Sucrose	91 ± 38	0.31 ± 0.04	−

[a] *P. putida* (pORFAGG1-951) was grown for 12 hr in BMM medium with carbon and nitrogen amendments as described in the text. Prior to harvesting of the cells, cell mass was monitored by OD_{570nm}.

[b] C23O-specific activity detected in extracts of cells grown in BMM supplemented media was converted to a percent of the C23O-specific activity detected in cells grown in unamended BMM medium. The values reported are the mean ± standard deviation of three separate experiments. The C23O-specific activity detected in BMM was 388.7 ± 83.4 nmoles/min/mg for the three experiments.

[c] Agglutinability reported is from incubation of the cells for 30 min with 1 mg/ml crude bean agglutinin.

The lack of correlation is not understood at present. This nutritional regulation of the *aggA* promoter differs from the *in vitro* expression of the *hrp* loci of plant pathogenic bacteria (Schulte and Bonas 1992; Willis *et al.* 1991). The *hrp* loci, which are involved in growth and survival of the bacteria in the leaf intercellular space, are expressed under minimal nutrient conditions but repressed under nutrient complex conditions (Willis *et al.* 1991).

One explanation for our observations concerning *aggA* gene expression is that this gene is not a regulatory locus for the agglutination phenotype. At least one other locus, termed *aggB,* essential for agglutination in *P. putida* has been identified and may be regulatory (Anderson *et al.* 1988; Buell and Anderson, unpublished). A second explanation is that additional events in stationary-phase cells may mask the agglutination phenotype, by modification or even physical covering of a receptor site. Thus the AGGA protein may continue to be produced but not be functional in the stationary-phase cells. A third explanation for *aggA* constitutive expression is that the AGGA protein provides another essential function in *P. putida* cells be-

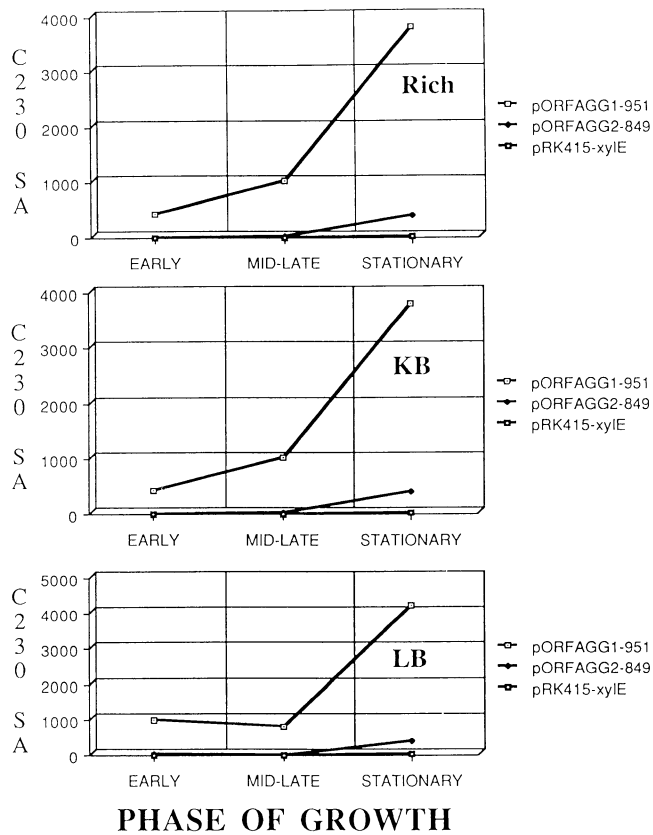

Fig. 4. Specific activity of C23O from early, mid-late logarithmic, and stationary-phase *Pseudomonas putida* transconjugants grown in rich, KB, and LB media. Transconjugant cells were grown to early logarithmic phase (OD_{570nm}: 0.16–0.24), mid-late logarithmic phase (OD_{570nm}: 0.93–1.43), or stationary phase (OD_{570nm}: 0.93–1.43) in rich, KB, or LB medium. Cells were washed twice in phosphate buffer, and assayed for agglutinability and C23O activity as described in the text. C23O activity is in nmoles product per minute per milligram of protein and is the mean of three experiments. All cells grown in the three media were agglutinable at early and mid-late logarithmic phase but were nonagglutinable at stationary phase.

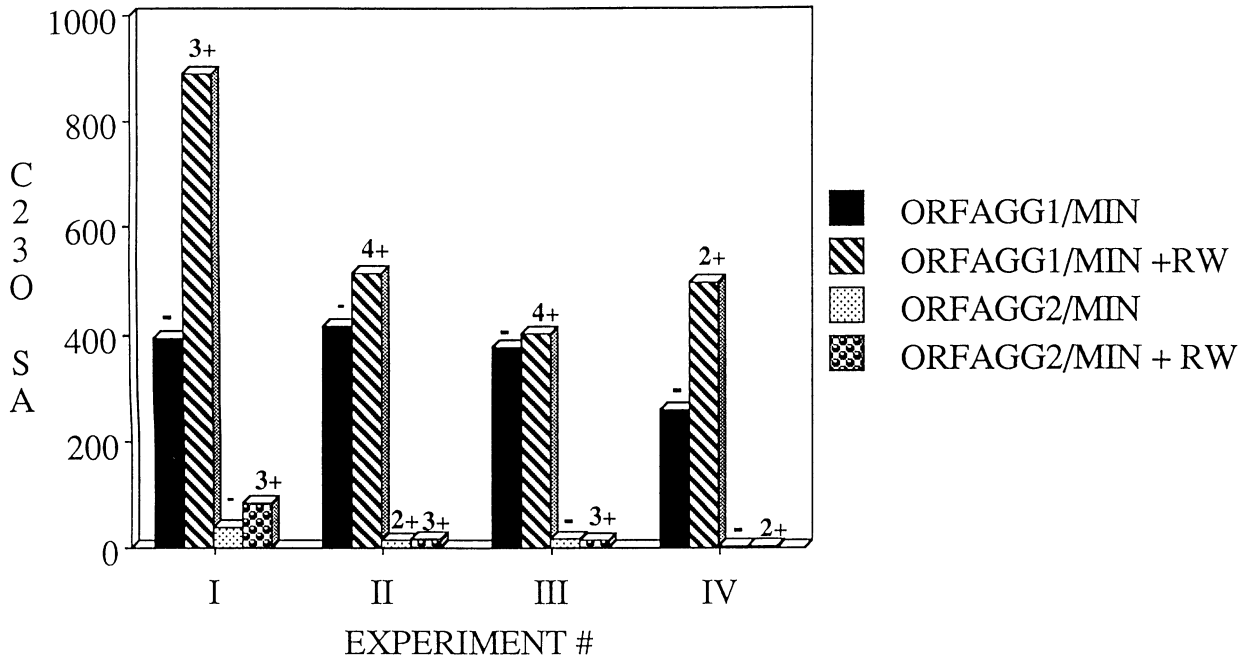

Fig. 5. Specific activity of C23O in *Pseudomonas putida* transconjugants grown in minimal or minimal medium supplemented with root components. Cells were grown for 12 hr and assayed for agglutinability and C23O activity as described in the text. The ORFAGG1 construct was pORFAGG1-951 and the ORFAGG2 construct was pORFAGG2-849. C23O specific activity is expressed as nmoles product per minute per milligram of protein. Agglutinability is noted above each bar. Cells grown in minimal supplemented with root wash medium grew in an agglutinated form in the medium and were self agglutinable upon assay. The results for four separate experiments (I–IV) are shown.

Table 2. C23O activity in *Pseudomonas putida* transconjugants recovered from the bean rhizosphere

	0 hr		48 hr		72 hr	
Treatment[a]	**C23O[b]**	**PR[c]**	**C23O**	**PR**	**C23O**	**PR**
Experiment 1						
P. putida (pORFAGG1-951)	1	0.44	3819	0.45	ND[d]	ND
P. putida (pORFAGG2-849)	0	0.34	330	0.33	ND	ND
P. putida (pRK415-*xylE*)	0	0.42	7	0.24	ND	ND
Phosphate buffer	0	0.06	4	0.04	ND	ND
Experiment 2						
P. putida (pORFAGG1-951)	0	0.43	923	0.34	748	0.42
P. putida (pORFAGG2-849)	0	0.37	217	0.35	2	0.30
P. putida (pRK415-*xylE*)	0	0.22	20	0.45	8	0.47
Phosphate buffer	0	0.11	0	0.08	0	0.10

[a] Roots of Dark Red Kidney bean were inoculated with *P. putida* transconjugants or treated with phosphate buffer and planted in vermiculite for 0, 48, or 72 hr as described in the text. Roots were washed of bacteria at given times, cells collected through centrifugation and extracts prepared as described in the text.
[b] C23O-specific activity is in nmol/min/mg.
[c] PR = total protein (mg) in cell extracts prepared from bacterial cells harvested from 30 seedlings as described in the text.
[d] Not determined.

sides agglutination, one that requires enhanced expression in stationary phase. A fourth possibility is that the C23O activity may not be a true reflection of transcription events from the *aggA* promoter. A high stability and low turnover time of the C23O protein in *P. putida* cells would be detected as a high specific activity in stationary-phase cells, as we observed. A similar situation is apparent when the reporter enzyme β-glucuronidase was used in *P. solanacearum*. High activity of β-glucuronidase was observed in extracts from stationary-phase cells containing fusions with genes involved in extracellular polysaccharide production (Cook and Sequeira 1991). In addition to processing of the C23O protein, there may be a copy number effect due to the use of a plasmid to carry the reporter construct. A higher copy number of the *aggA* promoter may titrate out controlling regulatory factors and produce anomalous expression. Future work using an AGGA specific antibody will provide information about turnover of the AGGA protein.

MATERIALS AND METHODS

Organisms and culture conditions.

Table 3 lists the bacterial strains and plasmids used in this study. All bacterial stocks were stored at −80° C in 15% glycerol and streaked to their respective medium as described below. *P. putida* isolate Corvallis (Anderson and Guerra 1985) was grown on King's medium B (KB; King *et al.* 1954) with nalidixic acid (50 μg/ml) and rifampin (50 μg/ml). The agglutination negative (Agg^-) mutant, 5123 (*aggA*::Tn*5*), derived from *P. putida* by Tn*5* transposon mutagenesis (Anderson *et al.* 1988), was streaked onto KB medium containing nalidixic acid, rifampin, and neomycin (50 μg/ml). Transconjugants of *P. putida* were grown on KB medium amended with nalidixic acid, rifampin, and tetracycline (75 μg/ml). *Escherichia coli* JM109 was grown on LB medium (Sambrook *et al.* 1989) amended with tetracycline (25 μg/ml). *E. coli* BL21(DE3) containing pET11 or pET-*aggA* derivatives was grown on M9 medium with carbenicillin (50 μg/ml; Sambrook *et al.* 1989). Magnesium sulfate was omitted from KB and LB media when tetracycline selection was employed.

Six media were employed to measure C23O activity *in vitro* with the *P. putida xylE* transconjugants: 1) minimal medium (Anderson and Jasalavich 1979; 60.3 mM K_2HPO_4, 33.1 mM KH_2PO_4, 1.7 mM sodium citrate, 7.6 mM $(NH_4)_2SO_4$, 5.8 mM sucrose, 1 mM $MgSO_4$), 2) rich medium (minimal medium supplemented with 0.5 [w/v] nutrient broth, 0.3 [w/v] yeast extract), 3) basal medium (BMM; minimal without sucrose), 4) KB, 5) LB, and 6) minimal medium supplemented with bean root wash medium. Minimal plus bean root wash medium was prepared by adding 1 mg of lyophilized Dark Red Kidney bean crude root wash (Anderson 1983) per milliliter of minimal medium, prior to autoclaving. BMM medium was amended with 1 mg/ml L-amino acids (methionine, histidine, glycine, aspartic acid, or lysine) or 20 mM carbon compounds (sucrose, D-mannitol, sodium citrate, sodium pyruvate, D-arabinose, D[+]galactose, or β-D-[−]fructose). Rich, KB, and LB liquid media were modified by addition of tetracycline (50 μg/ml) and omission of magnesium sulfate. Minimal, minimal plus root wash, and BMM medium contained magnesium sulfate and no tetracycline.

Agglutination assays.

Agglutinability was tested on *P. putida* cells grown in the various media as described above, washed twice in 50 mM potassium phosphate buffer (pH 7.5), and suspended in one-tenth volume of 50 mM potassium phosphate (pH 7.5). Bean root wash was prepared as described by Anderson (1983) from 7- to 10-day-old Dark Red Kidney bean plants (Idaho Bean Seed Co, Twin Falls, ID) grown in sterile vermiculite at 26° C. Crude bean root wash was suspended in water to a concentration of 100 mg/ml and particulate material was removed by centrifugation. Agglutination assays consisted of 900 μl of crude bean agglutinin (diluted to final concentrations of 10, 1, and 0.1 mg/ml) to which 100 μl of *P. putida* cells and 50 μl of 10 mM $MgCl_2$ was added. Control tubes were set up with no bean agglutinin to test for self agglutination of the cells. Agglutination tubes were shaken at 22° C, 120 rpm, and agglutinability was scored visually at 15 and 30 min. Maximal agglutination was rated from a 4+ to a − for no agglutination.

Generation of p*lac-aggA* constructs.

To provide further support for ORFAGG1 as the reading frame involved in agglutination, a fusion was made between the *lac* promoter of pRK415 (Keen *et al.* 1988) and an *aggA* deletion fragment which contains a promoterless ORFAGG1, as previously defined through sequence analysis (Buell and Anderson 1992). To make the chimeric *lac*-ORFAGG1 construct, p*lac*-ORFAGG1, the *aggA* insert present in pBAGG201Δ*Eco*RI E2 (Buell and Anderson 1992) was cloned into the *Kpn*I-*Xba*I sites of the broad host-range plasmid, pRK415, thus positioning the promoterless ORFAGG1 downstream of the *lac* promoter and forming a *lac*-ORFAGG1 transcriptional fusion (Fig. 1). p*lac*-ORFAGG1 has 23 bp 5′ of the ATG translational start site and all coding and 3′ noncoding regions of ORFAGG1 ligated into the polylinker region of pRK415. p*lac*-ORFAGG1 was introduced into the Agg^- mutant 5123 by triparental mating and tested for its ability to complement agglutination (Buell and Anderson 1992). A previously contructed clone, pRKAGG201Δ*Eco*RI E2, is 1,664 base pairs in length, contains a promoterless ORFAGG1, and is unable to complement agglutinability in the Agg^- mutant 5123 (Buell and Anderson 1992). This clone has ORFAGG2 oriented for transcription of ORFAGG2 mRNA downstream of the *lac* promoter of pRK415,

Table 3. Bacterial strains and plasmids used in this study

	Description[a]	Reference
Strain		
E. coli JM109	*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rec*A1Δ (*lac-pro*AB), [F′*tra*D36, *pro*AB, *lac*IqZΔM15]	Stratagene, Inc.[b]
Pseudomonas putida Corvallis		
Wild type	Nal^r, Rif^r, Agg^+	Anderson and Guerra 1985
5123	*aggA*::Tn*5*, Nal^r, Rif^r, Nm^r, Agg^-	Anderson *et al.* 1988
Plasmids		
pRK415	Vector; Tc^r	Keen *et al.* 1988
pET11	T7 transcriptional expression vector; Cb^r	Novagen[c]
pEPA53	Promoterless *xyl*E gene Ap^r; puc18	Cuskey and Sprenkle 1988
pRK415-*xylE*	Vector-*xylE*; Tc^r; $C23O^-$	This study
pBAGG201Δ*Eco*RI E2	1,664-bp *aggA*; 982-bp deletion of *Eco*RI terminus of *aggA*; promoterless ORFAGG1; $pBSKS^+$; Ap^r	Buell and Anderson 1992
pRKAGG201Δ*Eco*RI E2 = p*lac*-ORFAGG2	1,664-bp *aggA*; 982-bp deletion of *Eco*RI terminus of *aggA*; promoterless ORFAGG1; pRK415; Tc^r; *lac*-ORFAGG2 transcriptional fusion	Buell and Anderson 1992
p*lac*-ORFAGG1	1,664-bp *aggA*; 982-bp deletion of *Eco*RI terminus of *aggA*; promoterless ORFAGG1; pRK415; Tc^r; *lac*-ORFAGG1 transcriptional fusion	This study
pRKAGG201	Complete 2.7-kbp *aggA*; pRK415; Tc^r	Buell and Anderson 1992
pREVRKAGG201	Complete 2.7-kbp *aggA*; *aggA* in reverse orientation as in pRKAGG201; pRK415; Tc^r	Buell and Anderson 1992; This study
pT7-ORFAGG1	ORFAGG1 transcriptional fusion in pET11; Cb^r	This study
pT7-ORFAGG2	ORFAGG2 transcriptional fusion in pET11; Cb^r	This study

[a] Ap = ampicillin, Cb = carbenicillin, Nal = nalidixic acid, Nm = neomycin, Rif = rifampin, Tc = tetracycline.
[b] La Jolla, CA.
[c] Madison, WI.

forming a *lac*-ORFAGG2 transcriptional fusion. For the purposes of this study, this clone has been renamed p*lac*-ORFAGG2 to reflect the generation of a chimeric fusion of ORFAGG2 with the *lac* promoter of the vector pRK415 (Fig. 1).

Expression of the *aggA* locus in *E. coli*.

The *aggA* locus was examined in an *E. coli* expression system to detect potential protein products encoded by ORFAGG1 and ORFAGG2. A 1,664-bp fragment of *aggA,* containing a promoterlesss ORFAGG1, as predicted by sequence analysis, was isolated from pBAGG-201Δ*Eco*RI E2 (Buell and Anderson 1992) and ligated into the transcriptional expression vector, pET11 (Novagen, Madison, WI), such that the coding region of ORFAGG1 was positioned downstream of the T7 promoter. This construct, termed pT7-ORFAGG1, was transformed into *E. coli* BL21(DE3) expression cells (Novagen, Madison, WI). A second construct, pT7-ORFAGG2, with ORFAGG2 downstream of the T7 promoter also was made. *E. coli* BL21(DE3) cells were grown in M9 medium at 28° C at 200 rpm and transcription off the T7 promoter was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 30 min (Ausubel *et al.* 1989). After 2 hr in the presence of rifampin, cells were pulsed with ^{35}S-methionine for 5 min (Ausubel *et al.* 1989). Periplasmic proteins were obtained from labeled cells using an osmotic shock procedure (Ausubel *et al.* 1989). Total cell and periplasmic proteins were examined following separation on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli 1970). The gel was treated with Fluoro-Hance (Research Products International, Mt. Prospect, IL), dried, and exposed to X-Omat AR X-ray film (Eastman Kodak, Rochester, NY).

Generation of *aggA-xylE* constructs.

Fusions of ORFAGG1 or ORFAGG2 to the promoterless *xylE* reporter gene were made in the broad host-range plasmid pRK415 (Keen *et al.* 1988) using standard techniques as described in Sambrook *et al.* (1989) and Ausubel *et al.* (1989).

1) pRK415-*xylE.* The 2.0-kbp *Bam*HI-*Hin*dIII fragment of pEPA53 (Cuskey and Sprenkle 1988) containing the promoterless *P. putida xylE* gene was excised and ligated into *Bam*HI-*Hin*dIII restricted pRK415. The resulting construct, pRK415-*xylE,* generated a promoterless *xylE* gene in the opposite orientation of the *lac* promoter of pRK415 (Table 3).

2) ORFAGG1-*xylE* constructs. The *aggA* deletion clones pRKAGG201Δ*Hin*dIII A, C, E, G, H, I, J, K (Buell and Anderson 1992) contained sequential deletions of the *Hin*dIII terminus of the 2.7-kbp *Eco*RI-*Hin*dIII *aggA* locus in which the 3′ end of ORFAGG1 and the 5′ end of ORFAGG2 were deleted. These deletions ranged from 270 to 1,931 bp. Fusions of these clones to the promoterless *xylE* reporter gene were readily made by ligation of the 2.0-kbp *Bam*HI-*Hin*dIII *xylE* gene into the *Bam*HI and *Hin*dIII polylinker sites present in these clones (Fig. 3). These clones were oriented in the pRK415 vector such that ORFAGG1 was on the opposite strand as the *lac* promoter. pORFAGG1-1040 and pORFAGG1-951 were restricted with *Eco*RI and *Bgl*II, a unique restriction site in the clone, to release 852 bp of the *aggA* locus. The ends were filled in with Klenow, religated, and transformed into *E. coli* to yield pORFAGG1-188 and pORFAGG1-105 which contained 188 and 105 bp of the ORFAGG1 promoter, respectively (Fig. 3).

3) ORFAGG2-*xylE* constructs. The *aggA* deletion clones pBAGG201Δ*Eco*RI G, J, L, and M (Buell and Anderson 1992) contained sequential deletions of the *Eco*RI terminus of the 2.7-kbp *Eco*RI-*Hin*dIII *aggA* locus in which the 3′ end of ORFAGG2 and the 5′ end of ORFAGG1 were deleted. These deletions ranged from 1,384 to 2,303 bp. Inserts of these clones, obtained by restriction with *Hin*dIII and *Xba*I, were treated with Klenow to generate blunt ends. The blunt-ended inserts were then ligated into *Bam*HI-Klenow-alkaline phosphatase treated pEPA53, and ligation products were transformed into *E. coli* JM109. Positive clones with an ORFAGG2-*xylE* fusion were selected by restriction enzyme mapping. Inserts of these clones were isolated with an *Eco*RI-*Hin*dIII double digest, ligated into *Eco*RI-*Hin*dIII restricted pRK415, and transformed into *E. coli* JM109. To prevent any read through from the *lac* promoter, these clones, pORFAGG2-1262, -849, -551, and -343, were oriented such that the ORFAGG2-*xylE* fusion was in the opposite orientation with respect to the *lac* promoter (Fig. 3).

4) pORFAGG1-2646 and pORFAGG2-2646. The entire 2.7-kbp of the *aggA* locus was excised from pREVRK-AGG201 by restriction with *Eco*RI and ligated into the *Eco*RI site of pRK415-*xylE.* Clones were screened for fusion of the entire ORFAGG1 to *xylE* or ORFAGG2 to *xylE,* to yield pORFAGG1-2646 and pORFAGG2-2646, respectively (Fig. 3).

Generation of *P. putida* transconjugants.

Transconjugants of *P. putida* were made through triparental matings with wild-type *P. putida, E. coli aggA-xylE* fusion donors, and the helper *E. coli* (pRK2013) as described in Buell and Anderson (1992). Transconjugants were selected on KB medium containing nalidixic acid, rifampin, and tetracycline. To avoid interference of yellow pigmentation on KB medium, potential transconjugants were replica plated onto selective LB agar medium and tested for C23O activity by spraying colonies with a 1% catechol solution (Walter *et al.* 1987). Development of yellow coloration within minutes indicated colonies positive for C23O activity.

C23O activity assays.

P. putida transconjugants were grown in rich, KB, and LB media at 22° C, 150 rpm, to early logarithmic (OD_{570nm}: 0.16–0.24), mid-late logarithmic (OD_{570nm}:0.93–1.43), or stationary (OD_{570nm}:1.76–2.12) phase. For minimal and minimal plus root wash media, a culture of *P. putida* transconjugant cells were grown in minimal medium for 14–23 hr at 22° C at 150 rpm, harvested by centrifugation, and inoculated into flasks of minimal or minimal plus root wash media which were grown for 12 hr. For the BMM media amended with various carbon and nitrogen compounds, a starter culture was grown for 20–24 hr in BMM, collected through centrifugation, inoculated into

the BMM-amended media, and grown for 12 hr. Cells were harvested from all media by centrifugation and washed twice with 50 mM potassium phosphate (pH 7.5) buffer. An aliquot was assayed immediately for agglutinability. The remainder of the cell suspension was frozen at −80° C prior to determination of C23O activity. The frozen cell suspensions were thawed and lysed with a French pressure cell. Cell debris was removed through centrifugation (12,100 × *g*, 10 min), and the supernatant was assayed at 22° C immediately for C23O activity. C23O assays were performed spectrophotometrically at 375 nm as described in Gibson (1971). C23O activity is expressed in nanomoles product per minute per milligram of protein. Protein content of the lysate was measured through the Bio-Rad protein assay (Bio-Rad, Richmond, CA) using bovine serum albumin as the standard.

C23O activity *in planta*.

Dark Red Kidney beans, cultivar Camelot (Rogers Seed Co, Twin Falls ID), were soaked in water for 10 min, surface sterilized in 1% sodium hypochlorite for 20 min, and soaked again in water for 10 min prior to culling out imperfect seeds. This entire process was performed a second time using sterile water as the final rinse and seeds were planted into sterile vermiculite. For inoculation of roots with *P. putida* transconjugants, 7-day-old seedlings were removed from the vermiculite and adhering vermiculite was gently shaken off. Roots were dipped in a suspension of *P. putida* transconjugant cells (10^8–10^9 cfu/ml) which had been grown to logarithmic phase in rich medium, washed twice with 50 mM phosphate buffer (pH 7.5), and suspended in 50 mM potassium phosphate buffer (pH 7.5). Inoculated plants were replanted in fresh, sterile vermiculite. Seedlings (30 seedlings per time point) were harvested immediately (0 hr) or after 48 and 72 hr of incubation at 22° C. Cells were recovered from the rhizosphere by a 90-sec vigorous wash of the roots in 180 ml of sterile water to which 20 ml of 0.5 M potassium phosphate was added prior to decanting and centrifugation of the suspension. Cell pellets were suspended in 50 mM potassium phosphate buffer (pH 7.5) and frozen at −80° C. C23O activity was measured following French pressure treatment and clarification of the extract by centrifugation. Protein content was measured using the Bio-Rad assay (Bio-Rad, Richmond CA) using bovine serum albumin as the standard.

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