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# Differential Expression of Virulence Genes and Motility in *Ralstonia* (*Pseudomonas*) *solanacearum* during Exponential Growth

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**A complex network regulates virulence in *Ralstonia solanacearum* (formerly *Pseudomonas solanacearum*); central to this system is PhcA, a LysR-type transcriptional regulator. We report here that two PhcA-regulated virulence factors, endoglucanase (Egl) and acidic exopolysaccharide I (EPS I), and motility are expressed differentially during exponential growth in batch cultures. Tests with strains carrying *lacZ* fusions in a wild-type genetic background revealed that expression (on a per-cell basis) of *phcA* was constant but expression of *egl* and *epsB* increased 20- to 50-fold during multiplication from $1 \times 10^7$ to $5 \times 10^8$ CFU/ml. Expression of *xpsR*, an intermediate regulator downstream of PhcA in the regulatory cascade for *eps* expression, was similar to that of *epsB* and *egl*. Motility track photography revealed that all strains were essentially nonmotile at $10^6$ CFU/ml. As cell density increased, 30 to 50% of wild-type cells were motile between $10^7$ and $10^8$ CFU/ml, but this population was again nonmotile at $10^9$ CFU/ml. In contrast, about 60% of the cells of *phcB* and *phcA* mutants remained motile at $10^9$ CFU/ml. Expression of *phcB*, which is not positively regulated by PhcA, was the inverse of *epsB*, *egl*, and *xpsR* (i.e., it decreased 20-fold at high cell density). PhcB is essential for production of an extracellular factor, tentatively identified as 3-hydroxypalmitic acid methyl ester (3-OH PAME), that might act as an exponential-phase signal to activate motility or expression of virulence genes. However, growth of the *lacZ* fusion strains in medium containing excess 3-OH PAME did not result in motility or expression of virulence genes at dramatically lower cell densities, suggesting that 3-OH PAME is not the only factor controlling these traits.**

Recent work with *Ralstonia solanacearum* (previously *Pseudomonas solanacearum* [49]) revealed that this economically important phytopathogenic bacterium utilizes a complex, multicomponent regulatory system to control expression of virulence (23, 42) (Fig. 1). Among the genes in this network are *vsrAD*, *vsrBC*, *xpsR*, *phcBSR*, and *phcA*. VsrA (43) and VsrB (24) are membrane-bound proteins with amino acid sequence similarity to histidine kinase sensors of two-component systems for signal transduction. The partner components of VsrA and VsrB are VsrD and VsrC, respectively, which have amino acid sequences similar to those of DNA-binding response regulators. The central component of this network is PhcA, a member of the LysR family of transcriptional regulators (6, 41). Connecting PhcA and VsrA/VsrD to the regulation of *eps* expression is XpsR, an unusual regulatory protein that appears to work with VsrC to activate the *eps* promoter (23, 25). Mutation in *phcA* leads to decreased virulence, due in part to a 15-fold decrease in production of acidic extracellular polysaccharide I (EPS I) (7, 36), a 50-fold decrease in β-1,4-endoglucanase (Egl) activity (7), and a 10-fold decrease in pectin methylesterase (Pme) activity (44). Loss of PhcA also leads to about a 20-fold increase in endopolygalacturonase A (PglA) activity (44) and to increased motility (7). Previous studies indicated that *phcA* positively regulates itself; a 5- to 10-fold increase in expression occurs when a functional copy of *phcA* also is present in cells harboring a *phcA*::Tn*5lacZ* fusion (6). Wild-type *R. solanacearum* cells efficiently enter tomato and other host plants via roots, penetrate the vascular system, systemically colonize the xylem, and induce wilt symptoms (21). Tomato plants are not wilted by mutants lacking functional PhcA even when the mutants are inoculated directly into the vascular system (7), mainly due to the lack of EPS I (11, 21).

Expression of PhcA-regulated genes requires an endogenous extracellular factor (EF), tentatively identified as 3-hydroxypalmitic ($C_{16}$) acid methyl ester (3-OH PAME) (18), which is detectable both in culture supernatants and in the vapor phase above plate cultures (10). Production of the EF by *R. solanacearum* requires the *phcB* locus, which is located about 14 kb downstream from *phcA*. Nonpolar mutations in *phcB* result in a phenotype indistinguishable from that of a *phcA* mutant except that *phcB* mutants can be restored to the wild-type phenotype by addition of exogenous EF or 3-OH PAME (10, 18). Recently, we found that PhcS and PhcR, which are encoded by genes immediately downstream of *phcB*, form a putative two-component system for signal transduction that reduces expression of *phcA* in the absence of EF (9) (Fig. 1).

As part of their environmental response systems, numerous bacteria sense accumulation of endogenous extracellular signal molecules. For example, *Myxococcus xanthus*, *Enterococcus faecalis*, and *Streptomyces coelicolor* produce peptide or amino acid signal molecules that regulate fruiting body formation, conjugation, and aerial hyphae, respectively (26). However, probably the most common endogenous signal molecules are the family of acyl-homoserine lactones (acyl-HSLs) (reviewed in references 19 and 40). Many gram-negative bacteria make one or more structurally related acyl-HSLs to regulate traits as

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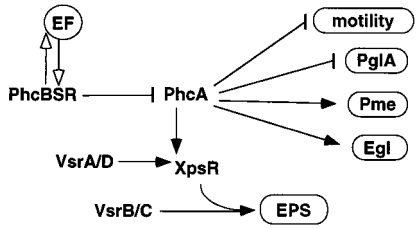


FIG. 1. Simplified model of the network regulating virulence factors and motility in *R. solanacearum*. Lines with filled arrowheads and bars represent positive and negative control, respectively, of gene expression. Lines with open arrowheads represent synthesis or sensing of the EF, which has tentatively been identified as 3-hydroxypalmitic acid methyl ester. PehS, PehR, and EpsR are not shown (reviewed in reference 42).

diverse as bioluminescence (*Vibrio fischeri*), conjugation (*Agrobacterium tumefaciens*), and production of extracellular enzymes (*Pseudomonas aeruginosa*). Based on work with *N*-(3-oxohexanoyl)-HSL (27), acyl-HSLs are thought to diffuse passively across bacterial membranes, which should result in the intracellular and extracellular concentrations being in equilibrium. The autoinduction paradigm states that transcription of regulated genes occurs when the threshold concentration of the extracellular signal compound (and thus the intracellular concentration) is exceeded. In general, this should occur only when cell density rapidly increases in a confined space; therefore, autoinducers are rationalized as a strategy by which bacteria can sense and respond to population density (quorum sensing) (19). Therefore, provided that no other regulatory systems are involved, addition of an autoinducer to a culture at low cell density should prematurely activate expression of autoinducible genes.

Production of the EF by wild-type *R. solanacearum*, and the ability of nonpolar *phcB* mutants to respond to it, suggested to us that this pathogen might exhibit population density-dependent expression of PhcA-regulated genes. We tested this hypothesis by monitoring batch cultures for expression of *lacZ* reporter fusions in selected PhcA-regulated genes and by examining motility microscopically. Finding that these traits were differentially expressed during exponential growth, we examined the effect of adding excess 3-OH PAME; its effect was slight, implying that one or more signals in addition to 3-OH PAME control PhcA-regulated gene expression and motility.

## MATERIALS AND METHODS

**Culture methods.** *R. solanacearum* strains (Table 1) were grown at 30°C in BG (1% Bacto Peptone, 0.1% Casamino Acids, 0.1% yeast extract, and 0.5% glucose), 1/10 BG (BG medium with the Bacto Peptone concentration reduced to 0.1%) or BGT agar (BG plus 1.6% agar and 0.005% tetrazolium chloride). EG broth (7) was used to culture cells for quantification of EPS I and exoenzyme activities. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium (35). When 3-OH PAME or PAME (Sigma Chemical Corp., St. Louis, Mo.) was added to cultures, hexane or methylene chloride-based stock solutions were diluted 1,000-fold into liquid media to give final concentrations of 15 to 20 nM and 3 to 5 mM, respectively. These concentrations fully restore *phcB* mutants to the wild-type phenotype for EPS I production and production of extracellular proteins including Egl, Pgl, and Pme (10, 17). Antibiotics used were ampicillin (100 μg/ml for *E. coli* and 10 μg/ml for *R. solanacearum*), kanamycin (50 μg/ml), nalidixic acid (20 μg/ml), spectinomycin (50 μg/ml), and tetracycline (15 μg/ml).

**Genetic manipulations and strain construction.** Isolation of and transformation with genomic DNA from *R. solanacearum* has been described (10). Plasmid construction and isolation, genetic transformation, and bacterial conjugations followed standard methods (8, 33). Strain AWG was created by random Tn*5lacZ* mutagenesis of strain AW following conjugal transfer of the suicide plasmid pSUP102-Gm::Tn*5*-B20 as previously described (14, 45). Although the gene mutated in AWG is unknown, the colony morphology of this strain is indistinguishable from that of the wild type, and it produced wild-type levels of EPS and Egl activity (data not shown). To make *R. solanacearum* strain AW-19A (*epsB*::Tn*5lacZ*) we first constructed pMT19 by cloning the 9-kb *Eco*RI fragment from cosmid pPF12 (11) into the ColE1 vector pTZ18U (34). pMT19 was mutagenized in *E. coli* DH5α with λTn*5*-B20 (45), plasmid DNA was electroporated into *R. solanacearum* (7), and an $EPS^-$, $LacZ^+$, kanamycin-resistant, and ampicillin-sensitive colony resulting from allelic replacement was chosen. Similarly, *R. solanacearum* AW1-28 (*egl-28*::Tn*5lacZ*) was constructed by allelic replacement using mutagenized pTD29 (39), followed by identification of an $Egl^-$, $LacZ^+$, kanamycin-resistant, and ampicillin-sensitive colony. AW1-88cm is a *cis*-merodiploid strain obtained by transforming AW1 with pGA91::Tn*5lacZ88* and selecting for resistance to kanamycin and ampicillin; unlike a *phcA* mutant, AW1-88cm is $EPS^+$. The *phcA*::Ω allele was moved into the genome of the wild type and various *R. solanacearum* mutants in one of two ways. In one method, ca. 5 μg of pGA952::Ω (13) DNA was added to a 10-ml culture of the strain to be mutated (optical density at 600 nm [$OD_{600}$] ≈ 0.1), and after shaking (250 rpm) at 30°C for 3 h, cells were harvested by centrifugation, suspended in 600 μl of BG, and spread on selection plates. ColE1 plasmids are unstable in *R. solanacearum*, and this method resulted in allelic replacement by homologous recombination at a frequency of $10^{-6}$ to $10^{-7}$ per cell plated; it also revealed that *R. solanacearum* is naturally competent for transformation by plasmid DNA. *phcA* mutants were identified by their nonmucoid colony type and tested for ampicillin sensitivity to check that the plasmid had not integrated into the chromosome, and their identity was confirmed by Southern blot analysis. Insertion of the *phcA*::Ω allele into AW1 resulted in strain AW1-PCΩ. In the second method (23), allelic replacement was achieved by genomic transformation with total DNA from strain AW1-PCΩ. To complement the *phcA*::Ω allele, wild-type *phcA* on pGA93 (7) was introduced by triparental mating; with the exception of the mutant carrying *epsB*::*lacZ*, the transconjugants were $EPS^+$.

**Assay of β-galactosidase activity.** For cultures grown to an $OD_{600}$ of ≈1.0, β-galactosidase assays were performed by using *o*-nitrophenyl-β-D-galactopyranoside as the substrate (35). Analysis of gene expression throughout a growth cycle was determined as follows. Exponential-phase cultures ($OD_{600}$ ≈ 0.5) were

TABLE 1. *R. solanacearum* strains

| Strain | Relevant characteristics[a] | Reference |
|---|---|---|
| AW and AW1 | Wild type and its $Nal^r$ sibling, respectively | 14 |
| AW1-83[b] | *phcB83*::Tn*5*+ (nonpolar); resembles AW1-80 but is inducible to wild type by 3-OH PAME; $Nal^r$ $Km^r$ $Ap^r$ $Cm^r$ | 10 |
| AW1-150 | *phcB150*::Tn*5lacZ* (nonpolar); inducible to wild type by 3-OH PAME; $Nal^r$ $Km^r$ | 9 |
| AW1-80 | *phcA80*::Tn*5* $Nal^r$ $Km^r$ | 6 |
| AW1-PCΩ | *phcA5*::Ω $Nal^r$ $Sp^r$ | This work |
| AW1-88cm | *phcA*/*phcA88*::Tn*5lacZ*, *cis*-merodiploid, $Nal^r$ $Km^r$ $Ap^r$ | This work |
| AW-19A | *epsB19*::Tn*5lacZ* $Km^r$ | This work |
| AW-19A-PCΩ | *epsB19*::Tn*5lacZ phcA*::Ω $Km^r$ $Sp^r$ | This work |
| AW1-28 | *egl-28*::Tn*5lacZ* $Nal^r$ $Km^r$ | This work |
| AW1-28-PCΩ | *egl-28*::Tn*5lacZ phcA*::Ω $Nal^r$ $Km^r$ $Sp^r$ | This work |
| AW-R165 | *xpsR165*::Tn*5lacZ* $Km^r$ | 23 |
| AW-R165-PCΩ | *xpsR165*::Tn*5lacZ phcA*::Ω $Km^r$ $Sp^r$ | This work |
| AWG | Random Tn*5lacZ* in uncharacterized gene, $Km^r$ | This work |

[a] $Nal^r$, $Km^r$, $Ap^r$, $Cm^r$, $Sp^r$, and $Tc^r$, nalidixic acid, kanamycin, ampicillin, chloramphenicol, spectinomycin, and tetracycline resistance, respectively.
[b] AW1-83 was generated by a transposition event where Tn*5*, the vector (pBR325), and IS*50* were inserted as a contiguous element, designated Tn*5*+.

TABLE 2. Effect of *phcA* on expression of various chromosomal genes containing Tn*5lacZ* fusions

| Strain | Genotype | β-Galactosidase activity[a] with: | |
|---|---|---|---|
| | | No plasmid | pGA93[b] |
| AW-19A | *epsB*::*lacZ* | 987 ± 53.5 | 580 ± 19.9 |
| AW-19A-PCΩ | *epsB*::*lacZ phcA*::Ω | 30 ± 3.0 | 666 ± 8.5 |
| AW1-28 | *egl*::*lacZ* | 201 ± 16.2 | 214 ± 13.7 |
| AW1-28-PCΩ | *egl*::*lacZ phcA*::Ω | 8 ± 0.87 | 149 ± 4.7 |
| AW-R165 | *xpsR*::*lacZ* | 573 ± 106.2 | 753 ± 16.3 |
| AW-R165-PCΩ | *xpsR*::*lacZ phcA*::Ω | 22 ± 1.4 | 959 ± 39.8 |
| AW1-150 | *phcB*::*lacZ* | 1,407 ± 87.0 | 1,100 ± 22.5 |
| AW1-150-PCΩ | *phcB*::*lacZ phcA*::Ω | 1,100 ± 101.0 | 796 ± 27.7 |

[a] Cells were grown to an $OD_{600}$ of ≈1.0, disrupted with sodium dodecyl sulfate-chloroform, and assayed (35). Values are in Miller units (means ± standard errors).

[b] pGA93 (7) carries a wild-type copy of *phcA* on a 2.2-kb fragment in pLAFR3, a low-copy-number vector.

diluted into fresh 1/10 BG broth (filtered to remove particulates greater than 0.25 μm in diameter, which makes later filtration steps easier) to give an initial density of $10^3$ to $10^4$ cells/ml and incubated at 30°C with shaking at 250 rpm. After about 10 h, samples of the culture (up to 100 ml) containing between $10^6$ and $10^9$ cells were removed at frequent intervals and filtered through 25-mm-diameter, 0.45-μm-pore-size nitrocellulose filters. The filters were cut into pieces (5 to 10 $mm^2$), which were placed into 1.0 ml of 10 mM Tris HCl buffer (pH 7.5) and frozen. After all samples were collected, they were thawed on ice and then sonicated while on ice to disrupt the bacteria. Cell lysates were assayed for β-galactosidase activity by using 0.25 mM methylumbelliferone-β-D-galactoside (Sigma Chemical Corp.) as the enzyme substrate. The concentration of fluorescent 4-methylumbelliferone released was determined with a model TKO 100 fluorimeter (Hoefer Scientific Instruments, San Francisco, Calif.) according to the manufacturer's instructions (Hoefer technical bulletin 129). Viable cell counts also were made at each time point to allow specific activity of β-galactosidase to be calculated as nanomoles of 4-methylumbelliferone per minute per $10^8$ CFU.

**Motility track photography.** Cultures were grown in 1/10 BG starting at about $10^4$ CFU/ml. After the cultures had reached about $5 \times 10^6$ CFU/ml, photomicrographs were taken every 1 to 2 h until early stationary phase ($10^9$ to $10^{10}$ CFU/ml) using pseudo-dark-field microscopy (magnification, ×100; 2-s exposure) (47). On photographic prints the motile cells appear as white streaks because they are moving during the exposure; the nonmotile cells are white dots. The average percent motile cells was calculated from at least three fields photographed at each time point. Samples also were dilution plated on BGT at each time point to determine the number of CFU per milliliter and to verify that wild-type cultures did not contain spontaneous *phcA* mutants (which are easily distinguished by their dark red color and nonmucoid colony morphology).

## RESULTS

**Regulation of virulence gene reporters by PhcA.** Previous results (23) obtained with spontaneous or Tn*5*-induced *phcA* mutants showed that PhcA positively regulates expression of *eps* and *xpsR*. To verify and quantify the involvement of PhcA in expression of the genomic *lacZ* fusions in *epsB*, *egl*, *xpsR*, and *phcB* that were to be used in subsequent time course studies, β-galactosidase activities were determined in wild-type and *phcA* mutant backgrounds from cells cultured to an $OD_{600}$ of ≈1.0 (about $10^9$ CFU/ml). PhcA positively regulated the *epsB*, *egl*, and *xpsR* fusions since inactivation of *phcA* by insertion of the Ω interposon reduced their expression to <4% of wild-type levels and a functional *phcA* in *trans* on pGA93 restored expression to near wild-type levels (Table 2). Under these conditions, production of β-galactosidase activity from *phcB*::Tn*5lacZ* was slightly reduced by inactivation or addition of *phcA* (Table 2). Inactivation of *phcA* in AWG reduced β-galactosidase activity expressed from the random *lacZ* fusion in this strain to about 30% of wild-type levels, and *phcA* in *trans* did not restore enzyme activity (data not shown). Because five other strains with random *lacZ* fusions exhibited similar reductions in β-galactosidase activity when *phcA*::Ω was introduced, this was judged to be nonspecific depression caused by the *phcA*::Ω allele.

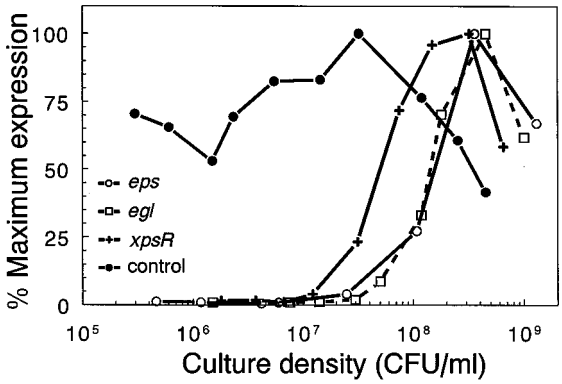


FIG. 2. Expression of PhcA-regulated genes in *R. solanacearum* during exponential growth. β-Galactosidase activity per cell was determined at intervals during batch culture of AW1-150 (*epsB*::*lacZ*), AW1-28 (*egl*::*lacZ*), and AW-R165 (*xpsR*::*lacZ*). Strain AWG, which contains a random *lacZ* fusion, was the control for gene expression not regulated by PhcA. Maximum β-galactosidase levels were 6.0 (*epsB*::*lacZ*), 0.46 (*egl*::*lacZ*), 12.8 (*xpsR*::*lacZ*), and 3.7 (AWG) nmol of 4-methylumbelliferone per min per $10^8$ CFU. The sensitivity threshold of this assay is ≤10 pmol of 4-methylumbelliferone or about $10^5$ cells of strain AWG. Representative data are shown; time course experiments were repeated at least once with similar results.

**Expression of virulence genes and motility during exponential growth.** When batch cultures were started at $10^4$ CFU/ml, 1/10 BG broth supported exponential multiplication (generation time = 1.0 to 1.4 h, based on viable-cell counts) of *R. solanacearum* wild-type and mutant strains for about 14 h, or until a density of $5 \times 10^8$ CFU/ml was reached (data not shown). During the subsequent transition to stationary phase, light-microscopic examination revealed that cells changed from rods to ellipses, a change similar to the reduction in the cell size of *E. coli* during the same transition (1, 30, 31). After 24 h, stationary-phase cultures contained more than $10^{10}$ CFU/ml.

To investigate whether transcription of *epsB*::*lacZ* and *egl*::*lacZ* fusions in *R. solanacearum* might be cell density-dependent we determined the amount of β-galactosidase activity per cell throughout exponential phase and the transition to stationary phase. In AW-19A expression of the *epsB*::*lacZ* fusion was very low before cultures reached $1 \times 10^7$ CFU/ml but β-galactosidase activity per cell increased about 100-fold before the cell density reached $5 \times 10^8$ CFU/ml (Fig. 2). As the cultures exited the exponential phase the β-galactosidase activity per cell decreased, likely due to a combination of decreased gene expression and smaller cell size. Experiments with AW1-28 (*egl*::*lacZ*) revealed similar patterns of gene expression (Fig. 2). Monitoring β-galactosidase activity in strain AWG demonstrated that our method reliably detected β-galactosidase specific activity at the lower cell densities and that expression of this random fusion did not fluctuate more than twofold under the culture conditions used (Fig. 2).

Cells from a young colony (2 days on BGT) of wild-type *R. solanacearum* are essentially nonmotile, whereas comparable cells of *phcA* or *phcB* mutant colonies are highly motile (7, 28). However, by starting batch cultures at $10^4$ CFU/ml and analyzing motility throughout the exponential phase, we found that a portion of the wild-type cells were transiently motile beginning at about $5 \times 10^6$ CFU/ml (Fig. 3A). The fraction of motile cells peaked at 30 to 50% near a cell density of $5 \times 10^7$ CFU/ml and subsequently declined to about 1% at $1 \times 10^9$

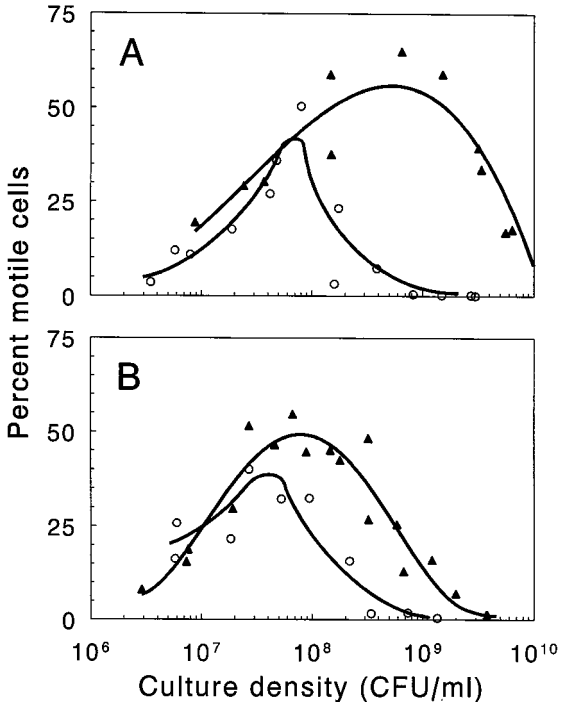


FIG. 3. Motility of *R. solanacearum* during exponential growth. Motility of cells removed from batch cultures was determined by using motility track photography. (A) Motility of the *phcB* mutant AW1-83 (▲) (the *phcA* mutant AW1-80 behaved similarly) and wild-type AW1 (○); (B) effect of adding PAME on motility of AW1-83 (▲) and AW1 (○). Shown are combined results of two time course experiments for each strain.

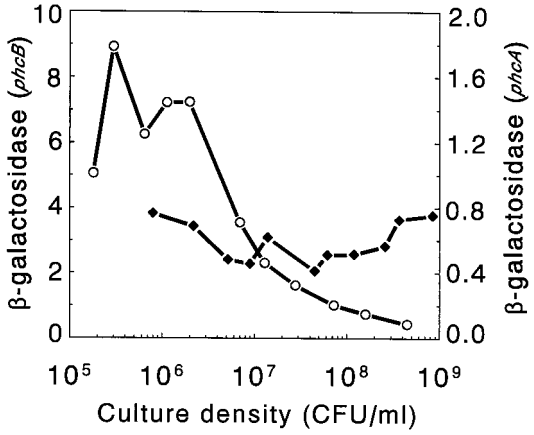


FIG. 4. Expression of *phcA* and *phcB* in *R. solanacearum* during exponential growth. The amount of β-galactosidase activity per $10^8$ CFU was determined at intervals during batch culture of AW1-88cm (*phcA*::*lacZ*) (◆) and AW1-150 (*phcB*::*lacZ*) (○). Representative data are shown; experiments were repeated at least once with similar results.

CFU/ml (Fig. 3A). The average speed for wild-type cells at $10^8$ CFU/ml was 29.9 μm/s (standard deviation ±4.1). *R. solanacearum* K60 and GMI1000 (reference 21 and references therein) also were transiently motile at densities near $10^8$ CFU/ml (data not shown). The decrease in motility of AW1 at higher densities was not due to increased viscosity resulting from EPS production because AW-19A, an $EPS^-$ strain, was transiently motile like the wild-type parent (data not shown). More importantly, the presence of motile cells in the wild-type culture was not due to the presence of spontaneous *phcA* mutants, because no $EPS^-$ colonies were recovered in the >1,000 CFU plated at each time point. The *phcA* and *phcB* mutants also were transiently motile, but unlike the wild type, 50 to 65% of the cells were still motile at $10^9$ CFU/ml (Fig. 3A). For these mutants, motility did not decrease until cell densities exceeded $10^9$ CFU/ml, presumably due to the gradual exhaustion of the proton motive force during the onset of stationary phase (2). These observations are consistent with a model in which expression of virulence genes and motility dramatically increase in response to cell densities of $\geq 10^6$ CFU/ml.

**Expression of regulatory genes during exponential growth.** XpsR, PhcA, and PhcB affect EPS production through a complex regulatory cascade (Fig. 1) (42). Similar to the results for the *eps* and *egl* fusions, expression of the *xpsR*::*lacZ* fusion in AW-R165 increased dramatically between about $1 \times 10^7$ and $4 \times 10^8$ CFU/ml and then began to decrease (Fig. 2). Because XpsR controls expression of the *eps* gene cluster (23), the differential expression of *epsB* probably reflects the expression of *xpsR*. To examine expression of the positively autoregulated *phcA* gene we used the *cis*-merodiploid mutant, AW1-88cm, which has a functional copy of *phcA* as well as a transcriptional *phcA*::*lacZ* reporter in the genome. During multiplication from $10^6$ to $10^9$ CFU/ml, the expression of *phcA*::*lacZ* stayed relatively constant, fluctuating twofold at the most (Fig. 4). The expression of *phcB* was the inverse of that observed for *egl*, *epsB*, and *xpsR*; at cell densities below $10^6$ CFU/ml *phcB* was abundantly expressed, and its expression decreased 10- to 20-fold by the time a density of $5 \times 10^8$ CFU/ml was reached (Fig. 4). Complementation with *phcB* in *trans* did not alter this pattern of gene expression (data not shown). Thus, from $10^6$ to $10^8$ CFU/ml, expression of *phcB* decreased, *xpsR* increased, and *phcA* was constant.

**Effect of 3-OH PAME on expression of PhcA-regulated genes.** The differential expression of PhcA-regulated genes during exponential phase could be explained by accumulation of an extracellular signal molecule. Indeed, the expression of *phcB* at low cell densities suggested that production of the EF (which requires *phcB*) should occur before PhcA-regulated genes are induced and supported the idea that accumulation of the EF might play a role in inducing expression of PhcA-regulated genes during exponential phase. If the EF (or 3-OH PAME) is the only limiting factor controlling expression of PhcA-regulated genes, then these genes should be induced soon after it is added to cultures at low cell densities.

We examined the effect of adding 3-OH PAME on expression of *xpsR* and *egl*, which are directly controlled by PhcA (23, 25). These assays were conducted by diluting exponential-phase cultures from about $4 \times 10^8$ to $1 \times 10^5$ CFU/ml, adding 3-OH PAME, and immediately collecting cells for β-galactosidase assays. Because the cells began with *xpsR* and *egl* being expressed, we expected them to be in a physiological state that is responsive to 3-OH PAME. When assayed in this manner, expression of *xpsR*::*lacZ* and *egl*::*lacZ* decreased in untreated cultures immediately after dilution, reached a minimum at about $2 \times 10^6$ CFU/ml for *xpsR* and about $1 \times 10^7$ CFU/ml for *egl*, and then began to increase. Expression of *egl*::*lacZ* in the culture treated with 3-OH PAME was shifted only slightly compared to the control (Fig. 5A). 3-OH PAME caused a more pronounced change in expression of *xpsR*, but the density at which expression began to increase was only one-third of that in the control (Fig. 5B). These results suggest that 3-OH PAME is not by itself responsible for increased expression of PhcA-regulated genes during exponential phase.

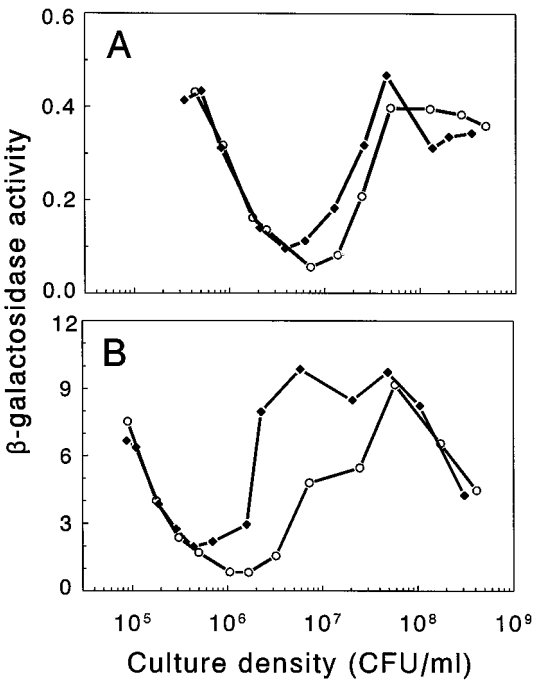


FIG. 5. Effect of 3-OH PAME on β-galactosidase activity (amount per $10^8$ CFU) expressed from PhcA-regulated gene in *R. solanacearum* during exponential growth. AW1-28 (*egl*::*lacZ*) (A) and AW-R165 (*xpsR*::*lacZ*) (B) were cultured in 1/10 BG (○) or in 1/10 BG plus 3-OH PAME (◆). Cultures were initiated with ca. $10^5$ cells. Representative data are shown; experiments were repeated at least once with similar results.

**Effect of PAME on motility.** At a final concentration of 5 mM, nonhydroxylated PAME can substitute for 3-OH PAME to restore a wild-type phenotype (10, 17). PAME did not shift the motility curve of the wild type or the *phcA*::Tn*5* mutant AW1-80 (data not shown), but it reduced about 10-fold the cell density at which the maximum percentage of *phcB* mutant AW1-83 cells were motile (Fig. 3B). Thus, AW1-83 provided with PAME had a motility curve intermediate between those of the wild type and untreated AW1-83.

## DISCUSSION

Stimulated by the autoinduction paradigm, we explored the possibility that expression of PhcA-regulated traits in *R. solanacearum* might be cell density dependent and, if so, whether 3-OH PAME was the critical extracellular signal in that process. By monitoring production of β-galactosidase per cell during batch culture, we found that three PhcA-regulated genes containing *lacZ* genomic fusions were differentially expressed during exponential growth. Expression increased sharply between $5 \times 10^6$ CFU/ml ($OD_{600} \approx 0.01$) and $5 \times 10^8$ CFU/ml ($OD_{600} \approx 0.5$), i.e., during the last 6 h of the true exponential phase in the medium used, and then began to decrease during the transition to stationary phase. This pattern of expression is clearly different from that of typical growth phase-regulated genes, which are activated by stress responses just before or during the transition to stationary phase ($OD_{600} \geq 0.5$) and often continue to be expressed in stationary phase (for examples, see references 30, 31, and 48). Therefore, changes associated with the transition to stationary phase may trigger the decrease in transcription of PhcA-regulated genes in *R. solanacearum*, but different factors or conditions likely are responsible for activating these genes during exponential phase.

One factor that might trigger the differential activation of PhcA-regulated genes in the exponential phase is accumulation of an intercellular signal molecule. A candidate molecule was the EF (or 3-OH PAME), since the *phcB* locus that is essential for EF production is abundantly expressed at cell densities below $10^6$ CFU/ml. However, addition of 3-OH PAME did not dramatically shift expression of PhcA-regulated genes in *R. solanacearum* to lower cell densities. This result contrasts with that obtained with *V. fischeri* or *Vibrio harveyi*, where adding exogenous acyl-HSL autoinducer stimulates bioluminescence at much lower cell densities (3, 15, 16), but is similar to that for *A. tumefaciens*, where addition of acyl-HSL autoinducer does not always stimulate conjugation because the autoinduction system is itself modulated by additional regulatory systems (25a). Therefore, it appears that some condition or factor in addition to extracellular 3-OH PAME is required for the activation of the PhcA-regulated genes and that this does not occur until cultures reach a density between $10^6$ and $10^7$ CFU/ml.

Another explanation for why 3-OH PAME did not more dramatically alter the density at which PhcA-regulated genes were activated might be that the complex medium used contains an inhibitory factor that must first be eliminated by bacterial metabolism (15). Two preliminary experiments, however, made this possibility appear unlikely. First, in a mineral salts medium, which presumably lacks any organic inhibitors, temporal expression of *egl*::*lacZ* was essentially the same as in standard 1/10 BG medium. Second, the effect of 3-OH PAME on transcription of *xpsR*::*lacZ* was the same in fresh medium and in conditioned medium consisting entirely of filter-sterilized 1/10 BG from a culture containing $8 \times 10^7$ CFU/ml of AW1-83, which makes no 3-OH PAME and should have metabolized any inhibitor.

There are several other possible signal molecules that might activate expression of virulence factors and motility in *R. solanacearum*. The most obvious possibility would be an acyl-HSL. Among plant-pathogenic bacteria, acyl-HSL-sensing systems that regulate exoenzyme production in *Erwinia carotovora* (38), EPS production in *Erwinia stewartii* (4), and conjugal transfer in *A. tumefaciens* (37) have been identified. *R. solanacearum* appears to make one or more acyl-HSLs (20), and we currently are investigating the role of these potential signal molecules. However, our results with conditioned medium and additional preliminary data suggest that acyl-HSLs are not important factors in the activation of PhcA-regulated virulence factors (17). Another potential signal molecule is the putative coinducer of PhcA that, by analogy to other members of the LysR family of transcriptional activators, probably is required for PhcA to activate transcription of target genes (41). However, a coinducer for PhcA has yet to be identified. Nevertheless, the observation that *phcA* expression is constitutive during exponential phase, but genes positively regulated by PhcA are induced 20- to 100-fold during the same time, is consistent with PhcA being inactive (or unstable) below $10^6$ CFU/ml and post-transcriptionally activated (or stabilized) at higher cell densities.

Under the conditions used, transient motility of wild-type *R. solanacearum* cells was exhibited entirely within the period of exponential growth. At low cell densities, motilities of a nonpolar *phcB* mutant (which does not make the EF) and a *phcA* mutant were similar to that of the wild type, indicating normal initiation of this process in the absence of these genes. Thus, as in the case of the PhcA-regulated virulence genes, the increase in motility of wild-type cells appears to be due to signals other than those associated with a change in growth phase. Since motility of *E. coli* also is initially low when cells are inoculated

into fresh medium (1) and is activated by increasing intracellular concentrations of cyclic AMP (32), the same may be true in *R. solanacearum*. In contrast to the wild-type *R. solanacearum* cells, *phcB* and *phcA* mutants remained highly motile until the transition to stationary phase at $10^9$ CFU/ml. Furthermore, addition of PAME to cultures of the *phcB* mutant largely eliminated this extended period of motility. These results suggest that a functional PhcA protein is responsible for the decrease in motility of wild-type cells that occurs during exponential phase. Very little is known about regulation of flagellation or motility in *R. solanacearum*, except that a two-component system encoded by *pehSR* appears to play a role in positively regulating motility (42). We have not investigated further the role of PhcA in these processes.

Our results resolved confusion in the literature as to whether *R. solanacearum* is motile. Bradbury (5) describes *R. solanacearum* as flagellated, and there are several reports of *R. solanacearum* cells becoming motile shortly after being transferred to distilled water or fresh medium (28, 46). However, due to the rapid occurrence of spontaneous $EPS^-$, motile, weakly virulent mutants (often due to inactivation of *phcA*), researchers have suspected that the motile cells that appeared in culture were mutants (28). More recently, Brumbley and Denny (7) and Yabuuchi et al. (49) concluded that wild-type *R. solanacearum* is nonflagellated and nonmotile. Our time course studies reconciled these conflicting conclusions by revealing that motility is transiently expressed in the absence of mutation. Preliminary electron microscopy of negatively stained *R. solanacearum* cells revealed that both the wild type and a spontaneous *phcA* mutant produced one to several polar flagella that were similar in appearance to those previously reported (22). It should be emphasized that to observe motility of wild-type *R. solanacearum* strains in broth, cultures should be started at densities below $10^5$ CFU/ml and cells should be observed before the culture reaches $10^8$ CFU/ml.

These results support our previous hypothesis (12) that *R. solanacearum* can alternate between two specialized phenotypes and that PhcA has a central role in this process. Above $10^8$ CFU/ml, the wild type has what could be considered a high-virulence phenotype. Production of high levels of EPS and of plant cell wall-degrading enzymes associated with this phenotype might be beneficial factors for colonization of host tissues or evading host defenses. At cell densities below $10^7$ CFU/ml the wild type presumably resembles a *phcA* mutant in being low in virulence, being motile, and producing low levels of EPS and certain plant cell wall-degrading enzymes; this phenotype might be well suited to survival in soil, a saprophytic existence, and/or early parasitic activities. It is clear from these observations that, especially when the early stages of pathogenesis are studied, it is important to consider the phenotype of the *R. solanacearum* cells used as the inoculum. Likewise, Klotz (29) noted the importance of the growth phase of the inoculum used for virulence and pathogenicity studies of the phytopathogen *Pseudomonas syringae*.

We predict that when *R. solanacearum* is not colonizing living plants (e.g., during existence in plant debris or in the soil) it exhibits the low-virulence, saprophytic phenotype and that during or soon after entry into a plant host, where bacteria can multiply rapidly to a high density, the high-virulence phenotype would be expressed. Whether *R. solanacearum* exhibits a transition from a saprophytic to a virulent phenotype in the rhizoplane or inside the plant is being investigated. A better understanding of the conditions and signals that promote a pathogenic or virulent behavior, or that enhance saprophytic survival, should lead to improved strategies for the prevention and treatment of bacterial diseases.

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