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Identification and Characterization of a Locus Which Regulates Multiple Functions in *Pseudomonas tolaasii*, the Cause of Brown Blotch Disease of *Agaricus bisporus*

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***Pseudomonas tolaasii*, the causal agent of brown blotch disease of *Agaricus bisporus*, spontaneously gives rise to morphologically distinct stable sectors, referred to as the phenotypic variant form, at the margins of the wild-type colonies. The phenotypic variant form is nonpathogenic and differs from the wild type in a range of biochemical and physiological characteristics. A genomic cosmid clone (pSISG29) from a wild-type *P. tolaasii* library was shown to be capable of restoring a range of characteristics of the phenotypic variant to those of the wild-type form, when present in *trans*. Subcloning and saturation mutagenesis analysis with Tn*5lacZ* localized a 3.0-kb region from pSISG29, designated the *pheN* locus, required for complementation of the phenotypic variant to the wild-type form. Marker exchange of the Tn*5lacZ*-mutagenized copy of the *pheN* locus into the wild-type strain demonstrated that a functional copy of the *pheN* gene is required to maintain the wild-type pathogenic phenotype and that loss of the *pheN* gene or its function results in conversion of the wild-type form to the phenotypic variant form. The *pheN* locus contained a 2,727-bp open reading frame encoding an 83-kDa protein. The predicted amino acid sequence of the PheN protein showed homology to the sensor and regulator domains of the conserved family of two component bacterial sensor regulator proteins. Southern hybridization analysis of *pheN* genes from the wild type and the phenotypic variant form revealed that DNA rearrangement occurs within the *pheN* locus during phenotypic variation. Analysis of *pheN* expression with a *pheN*::*lacZ* fusion demonstrated that expression is regulated by environmental factors. These results are related to a model for control for phenotypic variation in *P. tolaasii*.**

Pseudomonas tolaasii causes the economically important brown blotch disease of the mushroom *Agaricus bisporus* (Lange) Imbach (53). Synthesis of the low-molecular-weight (M_r, 1,985) lipodepsipeptide extracellular toxin tolaasin by *P. tolaasii* is primarily responsible for eliciting disease symptoms (6, 38, 39). Tolaasin causes disruption of cell membranes from a range of cell types and has both ion channel-forming and biosurfactant properties (6, 26, 38). The ability of *P. tolaasii* to show a positive chemotactic response to *A. bisporus* exudates (20) and its ability to attach to mycelial surfaces (9, 35, 37) may also be important as steps in the early stages of development of brown blotch disease.

P. tolaasii undergoes phenotypic variation, a strategy frequently used by bacteria to survive disparate environmental conditions. Old colonies of *P. tolaasii* frequently produce sectors which, when subcultured onto fresh medium, show different phenotypically distinct colonial forms (10, 29, 36). The wild-type strain is opaque, mucoid, pathogenic, and nonfluorescent, whereas the sectors are translucent, nonmucoid, nonpathogenic, and fluorescent (10, 57). In addition to alteration in virulence, qualitative analysis has revealed that a number of biochemical properties are also changed in the transition from the wild type to a phenotypic variant. These include the loss of the ability to produce tolaasin, hydrolyze casein, and grow normally on medium containing cetrimide (10). Finally, the variant form swims faster and shows a more rapid chemotactic response than the wild type (20). Phase change from the pathogenic to the nonpathogenic form in *P. tolaasii* has been referred to as a smooth-to-rough transition (10). This description generally designates a difference in the lipopolysaccharide (LPS) content of the two forms (16, 29). Recent studies by Hutchison (25) have demonstrated that there is no difference in the LPS profiles of the smooth and rough forms of *P. tolaasii*. Therefore, the terms "wild type" and "phenotypic variant" are used here instead of "smooth" and "rough," respectively.

The aim of this study was to identify the molecular switch controlling the transition from the wild type to the phenotypic variant in *P. tolaasii*. First, we quantitatively analyzed a range of phenotypic characteristics whose regulation is affected by transition from the wild type to the phenotypic variant form. Once identified, the ability of a cosmid library of the wild-type organism to convert the phenotypic variant to the wild-type phenotype was established. Saturation mutagenesis of the cloned DNA fragment and marker exchange of this genetic element in the wild-type form of *P. tolaasii* then allowed the range of phenotypic characteristics regulated by this genetic determinant to be identified. The genetic element involved in phenotypic switching was then cloned and sequenced, and its regulation of expression was studied.

(A preliminary account of this work was presented at the 7th International Symposium on Molecular Plant-Microbe Interactions [18].)

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MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The *Escherichia coli* and *P. tolaasii* strains used in this study are described in Table 1. *P. tolaasii* strains were grown on *Pseudomonas* agar F (PAF [56]) or in PB (Difco) liquid medium at

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
E. coli		
DH5α	*endA1 gyrSA96 hrdR17*(r_{K^-} m_{K^-}) *supE44 recA1*	Bethesda Research Laboratories, Inc., Gaithersburg, Md.
S 17-1	C600::RP4 2-(Tc::Mu)(Km::Tn*7*) Sm^r	45
P. tolaasii		
NCPPB1116S	Wild type; Phe^+ *met* Am^r	National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom
1116R	Spontaneous phenotypic variant; Phe^- *met* Am^r	This study
1116R(pSISG29)	NCPPB1116R carrying cosmid pSISG29; Phe^+ Am^r Tc^r	This study
1116R(pSISG3)	NCPPB1116R carrying cosmid pSISG3; Phe^+ Am^r Tc^r	This study
1116S-MR1	*pheN*::Tn*5lacZ* marker exchange mutant; Phe^- Am^r Kan^r	This study
P. reactans NCPPB387	Am^r Nx^r	National Collection of Plant Pathogenic Bacteria
Plasmids		
pLAFR3	IncP; *mob*$^+$ *cos*$^+$ Tc^r	48
pIJ3200	IncP; *mob*$^+$ *cos*$^+$ Tc^r	28
pRK2013	ColE1; *tra*$^+$ Km^r	14
pSISG29	pLAFR3 containing 29.9-kb *Sau*3A NCPPB1116S chromosomal DNA cloned into *Bam*HI	This study
pSISG16	pLAFR3 containing 16.8-kb fragment of DNA into *Bam*HI site obtained after partial digestion with *Pst*I	This study
pSISG9	9.4-kb *Sac*I fragment of DNA from pSISG9 cloned into *Sac*I site in pIJ3200	This study
pSISG5	pIJ3200 containing 5.6-kb fragment of DNA from pSISG9 cloned between *Bam*HI and *Sac*I sites	This study
pSISG3	pIJ3200 containing 3.4-kb fragment of DNA from pSISG5 cloned between *Xho*I and *Bam*HI sites	This study

28°C, and *E. coli* strains were grown in Luria broth (43) at 37°C. Recombinants were grown in the same media containing appropriate antibiotics at the following concentrations: kanamycin, 25 μg ml^{-1}; ampicillin, 100 μg ml^{-1}, tetracycline, 20 μg ml^{-1}, and nalidixic acid, 25 μg ml^{-1}. The wild-type strain of *P. tolaasii* (NCPPB 1116S) was obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom. A spontaneous ampicillin-resistant strain of *P. tolaasii* was selected after overnight growth of approximately 10^8 cells on PAF supplemented with ampicillin. A relatively stable typical phenotypic variant (designated 1116R) appearing in the form of translucent greenish-yellow sectors at the margins of wild-type colonies was isolated from a 5-day-old colony of 1116S cells grown on PAF at 28°C. This variant was subcultured two or three times from single colonies and was used for further studies. To determine the potential of the phenotypic variant form to spontaneously revert to the wild-type form, a single colony isolate of the phenotypic variant was inoculated into PB liquid medium and grown overnight at 28°C on a rotary shaker. Appropriate dilutions were plated onto PAF plates and incubated at 28°C, and the reversion frequency was calculated from the number of colonies showing the wild-type phenotype out of the total number of colonies. The terms "1116S" and "1116R" are used throughout to refer to the wild-type and phenotypic variant forms of *P. tolaasii*, respectively, and their phenotypes are designated Phe^+ and Phe^-, respectively.

DNA manipulations. Procedures for general DNA manipulation, including extraction of chromosomal DNA, restriction enzyme digestions, agarose gel electrophoresis, Southern blotting, plasmid isolation, and transformation of *E. coli* cells, have been described elsewhere (40, 43). Restriction endonucleases and a random priming kit were obtained from Boehringer Mannheim and Stratagene, respectively.

Cloning of a genetic region controlling phenotypic transition in *P. tolaasii*. A partial *Sau*3A genomic library constructed in broad-host-range cosmid vector pLAFR3 (48) was obtained from C. Brodey, Department of Plant Sciences, University of Cambridge. Recombinant cosmids in *E. coli* (ED8767), representing the wild-type genomic library of *P. tolaasii*, were mobilized en masse into 1116R grown from a freshly subcultured single isolated colony by triparental mating with the aid of the helper plasmid pRK2013 (15). The resulting transconjugants were initially screened visually, and colonies showing wild-type colony morphology (creamy white, opaque, and mucoid) were further checked qualitatively for the production of tolaasin and protease with the "white line in agar" and casein hydrolysis plate assays, respectively, as described below.

Individual recombinant cosmids from the colonies showing wild-type characteristics were isolated by the alkaline lysis method and back transformed into *E. coli* DH5α. The resultant *E. coli* colonies carrying cosmid clones were mated individually with *P. tolaasii* 1116R by triparental mating in order to analyze the ability of each clone to complement the recipient into Phe^+ transconjugants at a frequency approaching 100%. Transconjugants harboring different clones capable of converting >95% of the recipient cells into the Phe^+ form were selected and analyzed further.

Plasmid DNA prepared from the resulting transconjugants was digested to completion with three different restriction enzymes (*Eco*RI, *Hin*dIII, and *Pst*I) and separated electrophoretically on a 0.7% agarose gel. An identical restriction banding pattern in *Eco*RI, *Hin*dIII, and *Pst*I digests of all clones demonstrated the presence of a cosmid (pLAFR3) with the same 29.9-kb insert in each case, which was designated pSISG29. Relevant steps used to subclone the functional *pheN* locus from pSISG29 in pLAFR3 (48) and pIJ3200 (28) to an approximately 3.0-kb fragment of DNA (designated pSISG3) are indicated in Table 1 and Fig. 2. Transconjugants carrying pSISG29 and pSISG3 were characterized by quantitative assay for characteristics associated with the transition from wild type to phenotypic variant, including tolaasin synthesis, protease production, chemotaxis, motility, siderophore production, resistance to antibiotics, and growth rate.

Tn*5lacZ* mutagenesis of the cloned *pheN* locus. λ::Tn*5lacZ* mutagenesis was performed by the methods of de Bruijin and Lapuski (11) as modified by Huang et al. (23). The position of the transposon in pSISG9::Tn*5lacZ* isolated from individually picked transconjugants was checked by digestion with appropriate restriction enzymes and separation of the fragments on 0.7% agarose gels. Transposon insertions in plasmids are designated by "ins-" followed by letters (a, b, c, d, and i). Cosmids carrying Tn*5lacZ* in the 9.4-kb insert were introduced into the phenotypic variant form of *P. tolaasii* (1116R) to check their ability to convert 1116R to Phe^+.

Alternatively, *E. coli* S17-1 cells carrying pSISG9::Tn*5lacZ* were mated with *P. tolaasii* 1116R by triparental mating, and transconjugants were selected on PAF containing tetracycline, kanamycin, and ampicillin. Selection of recipients expressing Phe^- characteristics permitted the isolation of clones containing a mutant *pheN* locus as a result of Tn*5lacZ* insertion. Plasmid DNA from such clones was isolated and analyzed for the location of the transposon by restriction analysis.

Marker exchange mutagenesis of the *pheN* locus. Mutagenesis of the chromosomal *pheN* locus of wild-type *P. tolaasii* (1116S) was carried out with an adaptation of the method of Ruvkun and Ausubel (42). A cosmid clone, pSISG5/ins-i, carrying a 5.5-kb insert with a Tn*5lacZ* insertion in the *pheN* locus, was linearized by digestion with the restriction enzyme *Kpn*I (a unique site in pSISG5/ins-i) and introduced into *P. tolaasii* 1116S by electroporation (19). Marker exchange mutants arising by double homologous recombination were identified by screening individual kanamycin-resistant colonies for spontaneous loss of pIJ3200 on the basis of tetracycline sensitivity. Potential marker exchange mutants were verified by Southern hybridization. A marker exchange mutant of *P. tolaasii* 1116S-M1 (*pheN*::Tn*5lacZ*) was characterized by qualitatively and quantitatively assaying

characteristics associated with phenotypic variation in *P. tolaasii* as described below.

To determine the direction of transcription of the *pheN* locus, transconjugants carrying cosmid clones with Tn*5lacZ* inserted into the *pheN* locus in either orientation were plated separately onto PAF supplemented with tetracycline, kanamycin, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [80 μg ml^{-1}]). The plates were incubated at 28°C for 48 h, and the direction of Tn*5lacZ* in the clones showing blue colonies because of expression of the promoterless *lacZ* gene was deduced to be the direction of transcription of the *pheN* locus.

Measurement of characteristics associated with transition from wild type to phenotypic variant. The following tests of the *P. tolaasii* wild type (1116S), the phenotypic variant (1116R), 1116R carrying pSISG29 or pSISG3, and 1116S-M1 were performed to determine qualitatively and quantitatively the degree of similarity between these strains.

(i) Tolaasin synthesis. Qualitative measurement of tolaasin production by different *P. tolaasii* isolates was performed with the white line in agar test and the rapid pitting test described elsewhere (56). For quantitative measurements of tolaasin, 10-ml volumes of PB liquid medium (PB plus appropriate antibiotics for cultures carrying plasmid) in 25-ml flasks were inoculated with the bacterial cultures and incubated at 28°C with vigorous shaking until an optical density at 600 nm (OD_{600}) of 1.0 was reached. Two milliliters of the cultures was transferred to sterile Eppendorf tubes, and the bacteria were removed by centrifugation (10,000 × *g*, 2 min, room temperature). Impurities from the supernatants were removed with Sep-Pak cartridges (Millipore) and equilibrated with acetonitrile and then with 0.1% (vol/vol) trifluoroacetic acid. After loading of the sample onto the cartridge at a constant flow rate of 2 ml min^{-1}, partially purified tolaasin was eluted with 5 ml of 90% acetonitrile–0.1% trifluoroacetic acid. One milliliter of the eluted sample was analyzed by reverse-phase high-performance liquid chromatography (HPLC) as described by Hutchison and Johnstone (26). Tolaasin eluted at 60.1% (vol/vol) acetonitrile, and its concentration in the sample was quantified by comparison of the peak size with those of HPLC-purified tolaasin standards.

(ii) Casein hydrolysis. The ability of *P. tolaasii* isolates to hydrolyze casein was established essentially as described by Cutri et al. (10). Cultures were spotted onto PAF containing 5% (wt/vol) dried skim milk and incubated at 28°C for 24 to 48 h. Zones of clearing indicated extracellular protease production. To quantify the amount of extracellular protease produced by different strains, a method based on hydrolysis of fluorescein isothiocyanate (FITC)-casein (54) was used. A 10-ml volume of PB liquid medium in 25-ml flasks was inoculated and grown at 28°C with vigorous shaking until an OD_{600} of 1.0 was reached. One milliliter of the culture was transferred to a sterile Eppendorf tube, and the cells were removed by centrifugation (10,000 × *g*, 2 min, 4°C). The supernatant was sterilized by being passed through a sterile 0.22-μm-pore-size filter (Sartorius) and was stored at 4°C. Triplicate 10-μl samples of an appropriate dilution of the supernatant, 20 μl of buffer (20 mM Tris-HCl [pH 8.5]), and 20 μl of FITC-casein (5 mg ml^{-1}) were mixed in a sterile tube and incubated at 37°C for 16 h. The reaction was stopped by addition of 120 μl of 5% (wt/vol) trichloroacetic acid. After incubation for 1 h at room temperature, the samples were centrifuged (8,000 × *g*, 5 min, room temperature). A total of 30 μl of the supernatant was mixed with 200 μl of 50 mM Tris-HCl (pH 8.5) and dispensed into the wells of a microtiter plate. FITC-casein release was determined with a fluorescence spectrometer (Perkin-Elmer) with a slit width of 5 nm and excitation and emission wavelengths set at 490 and 525 nm, respectively (54). Relative enzyme activity was calculated by comparing the fluorimeter readings with trypsin used as a standard. One unit of extracellular protease was defined as the amount of enzyme required to release the same amount of FITC from FITC-casein as 1 ng of trypsin after 16 h at 37°C.

(iii) Fluorescent pigments. To assess the production of fluorescent pigments, cultures of *P. tolaasii* were grown in PB liquid medium to an OD_{600} of 2.0 and the cells were removed by centrifugation (10,000 × *g*, 2 min, 4°C). The supernatants were sterilized by being passed through 0.22-μm-pore-size filters (Sartorius). Fluorescent pigments were measured by scanning the emission spectrum of the supernatant against a PB liquid medium blank from 400 to 600 nm with a Perkin-Elmer fluorescence spectrometer with a slit width of 5 nm and an excitation wavelength of 390 nm (12). The relative concentration of fluorescent pigments in different samples was determined from the difference in emission peak heights at 460 nm.

(iv) Growth rate. One-liter flasks containing 250 ml of PB were incubated overnight at 28°C in an orbital incubator shaker (200 rpm) to ensure good aeration of the medium. The flasks were inoculated to an OD_{600} of 0.01, and the specific growth constant was determined from a plot of ln OD_{600} against time. Transconjugants carrying cosmid clones pSISG29 and pSISG3 were grown in PB liquid medium supplemented with tetracycline (20 μg ml^{-1}).

(v) Chemotaxis and motility. The effects of time and concentration of attractant on chemotactic behavior with the capillary assay were studied as described by Grewal and Rainey (20). Computerized motion analysis of free-swimming cells to determine mean speed and mean run length was carried out as described by Packer and Armitage (33).

(vi) Antibiotic resistance. Bacterial cultures were grown overnight in PB liquid medium and diluted to an OD_{600} of 0.01 in fresh PB liquid medium. One hundred microliters of cells was pipetted into each well of sterilized disposable microtiter plates with a multipipette. Antibiotics dissolved in appropriate solvent were added in a twofold serial dilution, and the plates were incubated at 28°C for 24 h. The concentration of inhibitory compounds in the first well which showed no visible bacterial growth was recorded as the MIC.

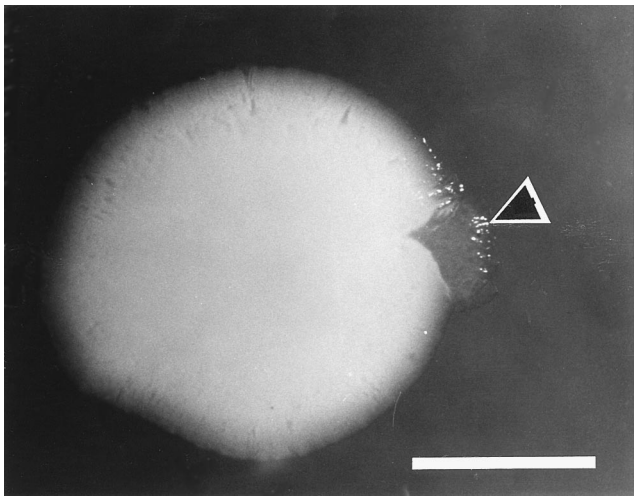

FIG. 1. Growth of *P. tolaasii* on PAF medium after 7 days at 25°C, showing the wild-type (1116S; Phe^+, white and opaque) and phenotypic variant (1116R, Phe^-, greenish, and translucent [arrowhead]) forms. Bar, 2 mm.

DNA sequencing. DNA fragments to be sequenced were cloned into *Bam*HI-*Xho*I sites in the polylinker of vector pBluescript (Stratagene), and double-stranded DNA sequencing on both strands was performed by the chain termination method of Sanger et al. (44) with a Sequenase version 2.0 sequencing kit (United States Biochemical Corp.). Sequencing primers (17-mer) were synthesized by the SERC Cambridge Centre for Molecular Recognition at the University of Cambridge. In case of band compression problems caused by the high G+C content of *P. tolaasii*, dGTP was substituted for by its analog dITP. DNA sequence analysis and deduced amino acid analysis were carried out with the Staden (47) and Genetics Computer Group (University of Wisconsin) software packages.

Gene expression assays. The level of expression of the *pheN* gene was determined with *pheN*::*lacZ* gene fusions by measuring the levels of β-galactosidase activity. *P. tolaasii* 1116S-M1 was grown overnight at 25°C in PB liquid medium containing kanamycin (20 μg ml^{-1}), except where otherwise stated. For plate assays, *P. tolaasii* 1116S-M1 was inoculated onto PAF plates containing 80 μg of X-Gal ml^{-1} and incubated at 25°C for 48 h. In liquid culture experiments, the cell densities of *P. tolaasii* 1116S-M1 inocula were adjusted to an OD_{600} of 0.1, and 20 μl was used to inoculate 200 ml of PB medium. Flasks were placed in a shaking incubator at 25°C, and the OD_{600} of aliquots removed after different time intervals was measured. β-Galactosidase activity in the aliquots was measured essentially as described by Miller (31). A blank was prepared by addition of PB medium instead of bacterial culture. Three replicates for each sample were assayed. Spent culture waste of the wild type (1116S) was prepared as follows. Five hundred milliliters of PB liquid medium was inoculated with a loopful of bacterial culture and incubated at 25°C on a rotary shaker (300 rpm) for 48 h. The cells were removed by centrifugation (7,500 × *g*, 15 min, room temperature), and the supernatant was filtered through Whatman filter paper no. 1. The pH of the culture supernatant was adjusted to 7.3, and the supernatant was autoclaved for 15 min at 121°C.

Nucleotide sequence accession number. The nucleotide sequence of the *pheN* gene has been deposited with GenBank under accession number U25692.

RESULTS

Characterization and comparison of *P. tolaasii* strains. Single cell isolates of 1116S produce domed, opaque, nonfluorescent, and mucoid colonies from which a phenotypic variant, designated 1116R, appearing as a sector with flat, translucent, and fluorescent colonies (Fig. 1), was selected and used for further analysis. Both strains were characterized by quantitatively examining their stability as well as their toxin production, biochemical characteristics, swarming behavior, and pathogenicity on mushrooms (Table 2). These characteristics were found to conform in all respects to those shown by previous

TABLE 2. Comparison of characteristics of *P. tolaasii* strains

Characteristic[a]	1116S	1116R	1116R(pSISG29)	1116R(pSISG3)	1116S-MR1
Protease (relative enzyme activity)[b]	324 ± 13	2 ± 3	281 ± 15	270 ± 13	2 ± 3
Tolaasin (μg ml^{-1})	250	<1	216	200	<1
Fluorescent pigment (relative fluorescence at 460 nm)	42.4	603	103	121	684
Mean swim speed (μm s^{-1})	2.1 ± 0.5	10.0 ± 0.9	4.1 ± 0.7	3.9 ± 0.2	12.5 ± 1.8
Mean run length (μm)	2.5 ± 0.3	11.6 ± 1.6	3.9 ± 0.7	4.0 ± 1.0	10.1 ± 1.6
MIC of polymyxin B (μg ml^{-1})	12.5	0.4	12.5	12.5	0.2
MIC of nalidixic acid (μg ml^{-1})	0.8	3.15	1.5	1.5	6.25
Specific growth rate constant (h^{-1})	0.70	1.2	0.72	0.86	1.2

[a] 1116S, 1116R, 1116R(pSISG29), 1116R(pSISG3), and 1116S-MR1 were grown in PB liquid medium at 28°C, and the tests were carried out as described in Materials and Methods.
[b] One unit of enzyme activity is defined as the amount of activity equal to that of 1 ng of trypsin in the FITC-casein assay described in Materials and Methods.

qualitative analysis (10, 57). The spontaneous phenotypic variant 1116R differed from the wild type in its inability to synthesize tolaasin or to hydrolyze casein as well as its ability to synthesize larger amounts of fluorescent pigments, to swim faster, and to grow more rapidly than 1116S. In addition, 1116R was more sensitive to both the membrane-acting antibiotic polymyxin and the intracellular antibiotic nalidixic acid. Analysis for the presence of plasmid DNA revealed that no indigenous plasmids were present in either 1116R or 1116S strains of *P. tolaasii*. Reversion of 1116R to 1116S at a frequency of approximately 1 in 10^6 cells was observed when an actively growing single cell isolate of 1116R was analyzed.

Cloning of the *pheN* locus involved in phenotypic variation of *P. tolaasii*. A cosmid bank of 1116S chromosomal DNA in the broad-host-range vector pLAFR3 was transferred en masse by conjugation to 1116R. A total of 3,000 transconjugants were screened for colonies showing a wild-type colony morphology, tolaasin production, and ability to hydrolyze casein. Cosmid clones isolated from these transconjugants were introduced individually in 1116R to test for restoration of the wild-type phenotype. Six out of the resulting 10 clones isolated were capable of reverting virtually 100% of the 1116R population to Phe^+. An identical restriction banding pattern for enzymes *Eco*RI, *Hin*dIII, and *Pst*I in all six clones (results not shown) demonstrated the presence of a cosmid with the same 29.9-kb insert in each case, which was designated pSISG29. Further confirmation of the ability of pSISG29 to complement the phenotypic variant to Phe^+ was revealed by quantitatively comparing the physiological and biochemical characteristics of 1116R(pSISG29) merodiploids with those of the 1116S and 1116R forms. This analysis revealed that the 1116R(pSISG29) merodiploid was identical to 1116S in all respects (Table 2). It was also shown that the maximum chemotaxis response time and the optimal chemoattractant concentration of Casamino Acids (20) for the 1116R(pSISG29) merodiploid were identical to those of 1116S (results not shown). Finally the antibiotic sensitivities to kanamycin, ampicillin, erythromycin, streptomycin, and gentamicin of the 1116R(pSISG29) merodiploid were also the same as those of 1116S (results not shown).

Restriction mapping and subcloning. A restriction enzyme map of pSISG29 (pLAFR3 containing a 29.9-kb insert of 1116S chromosomal DNA) was determined (Fig. 2). In order to subclone the *pheN* locus, a partial digest of the 29.9-kb insert DNA with *Pst*I was used to construct a minilibrary, and a 16.8-kb fragment of DNA (pSISG16) was found to be capable of converting 1116R to 1116S. Further subcloning of the *pheN*-containing region was accomplished by cutting a 9.4-kb *Sac*I fragment from pSISG16 and ligating it into pIJ3200 (28) to create pSISG9. Conjugation of pSISG9 into 1116R resulted in the same phenotype as that exhibited by the functional pSISG29 and pSISG16 cosmids. To determine the boundaries of the *pheN* locus as well as to establish the direction of transcription, saturation transposon mutagenesis was used. After mutagenesis of pSISG9 with Tn*5lacZ*, the locations of the transposon insertions were determined by restriction mapping. Since Tn*5lacZ* contains a *Bam*HI site at its left end (46), a 5.6-kb *Bam*HI-*Sac*I fragment was isolated from pSISG9:: Tn*5lacZ*/ins-d and cloned into pIJ3200 to form pSISG5. Transfer of pSISG5 to 1116R yielded transconjugants with a wild-type colony morphology as well as the ability to synthesize tolaasin and hydrolyze casein, indicating the presence of a functional *pheN* locus. Characterization of pSISG5 by saturation mutagenesis was carried out, and sites of relevant insertions together with insertions in plasmid pSISG9 are shown in Fig. 2. Inactivation of the *pheN* locus was analyzed by mating each mutated cosmid individually into 1116R and testing whether transconjugants showed wild-type characteristics. Transposon insertions which inactivated the *pheN* locus were found within a 2.9-kb region between ins-b and ins-d. A total of three different transposon insertions were mapped within the 2.9-kb region, all of which failed to convert 1116R into 1116S, while insertions in the flanking regions did not prevent complementation by the *pheN* locus. The *Xho*I site on the right end of Tn*5lacZ* in plasmid pSISG5::Tn*5lacZ*/ins-b facilitated the isolation of a 3.4-kb *Xho*I-*Bam*HI fragment, which was subcloned into pIJ3200 to form pSISG3. Analysis of the ability of pSISG3 to complement 1116R to Phe^+ showed that only 30% of the population of transconjugants instead of the expected 95 to 100% (as demonstrated by pSISG29, pSISG16, pSISG9, and pSISG5) expressed wild-type characteristics. Transconjugants harboring pSISG3 were tested quantitatively for characteristics associated with phenotypic variation, and results very similar to those with 1116R(pSISG29) were obtained (Table 2), demonstrating the ability of pSISG3 to complement 1116R to Phe^+. Furthermore, if the merodiploid strain (1116R carrying pSISG29 or pSISG3) was streaked onto medium lacking tetracycline, Phe^- outgrowths arose at a high frequency (approximately 50% in the population) at the margins of the colonies. These outgrowths were shown to be tetracycline sensitive and to have lost pSISG3. Thus, restoration of the wild-type phenotype was not due to spontaneous reversion but to an effect which occurred in *trans*.

Determination of direction of transcription of the *pheN* locus. The direction of transcription of the *pheN* locus was determined by mutating the *pheN* locus with Tn*5lacZ* and analyzing β-galactosidase activity from the promoterless *lacZ* gene within Tn*5lacZ*. Colonies of 1116R containing a Tn*5lacZ*-mutated cosmid were grown on PAF plus X-Gal and scored for white or blue colonies. The ins-i insertion within the *pheN* locus showed blue colonies caused by expression of the *lacZ*

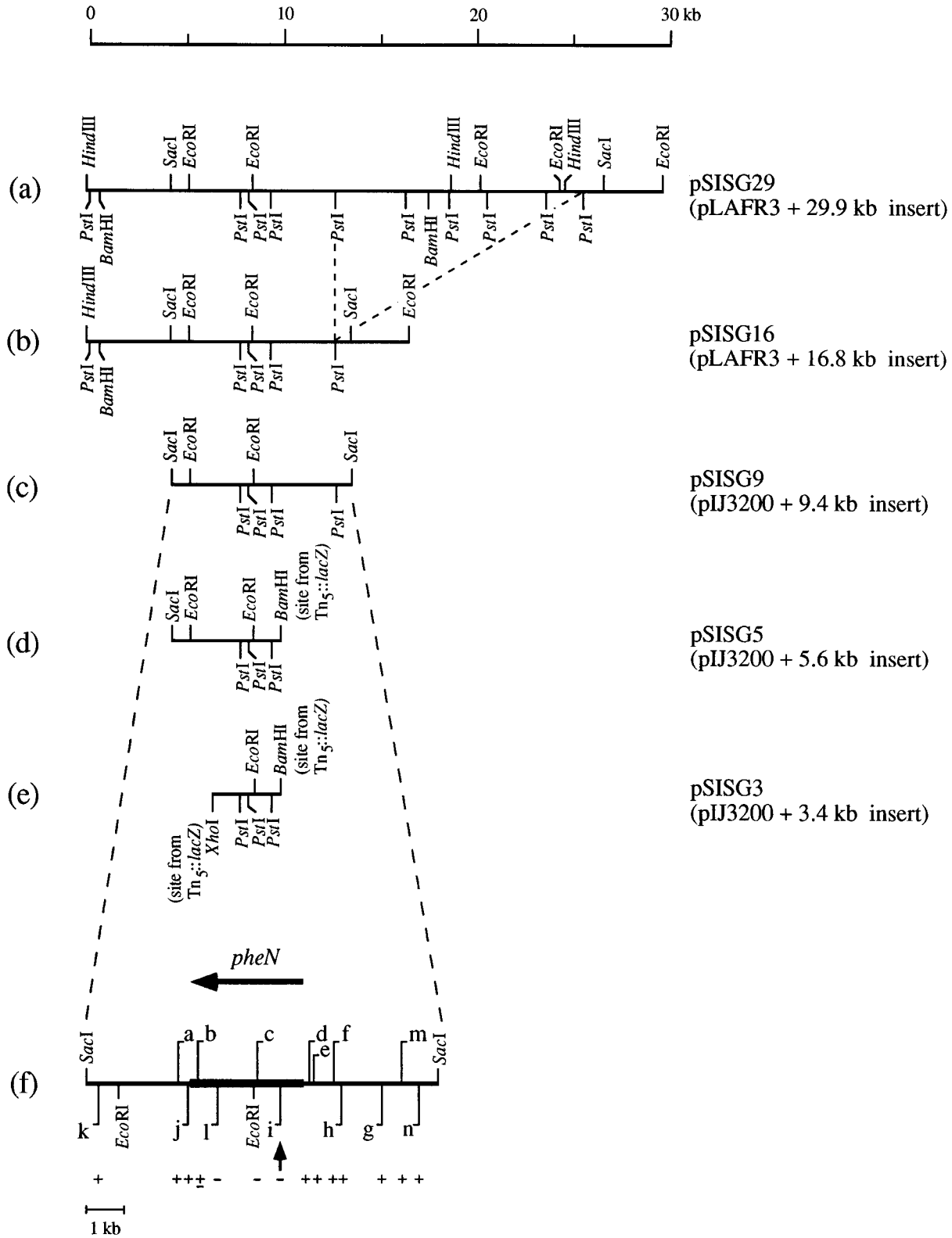

FIG. 2. Strategy for subcloning and results of Tn*5lacZ* saturation mutagenesis analysis of the *pheN* locus controlling phenotypic switching in *P. tolaasii*. (a) Restriction map of the 29.9-kb region of 1116S chromosomal DNA containing the *pheN* locus, cloned in cosmid pLAFR3 (pSISG29). (b to e) Sequential subcloning of the *pheN* functional unit with different restriction enzymes. Tn*5lacZ* was used to artificially generate additional restriction enzyme sites for subcloning of the 5.6-kb (pSISG5) and 3.4-kb (pSISG3) fragments containing the *pheN* locus. (f) Insertion sites of transposon Tn*5lacZ* in the *pheN* locus are shown by vertical bars, and the orientation of the promoterless *lacZ* gene is indicated by the flag on each bar. −, inactivation of the *pheN* gene in *trans*; +, an insertion in *trans* at this position did not inactivate the *pheN* gene. The vertical arrow indicates the insertion which was marker exchanged into the chromosome. The horizontal arrow indicates the approximate location and direction of transcription of the *pheN* locus.

gene. Insertion in the opposite orientation in the *pheN* region (ins-c [Fig. 2]) did not result in detectable expression of β-galactosidase. The deduced direction of transcription of the *pheN* locus is shown in Fig. 2.

Characterization of the marker exchange mutant. To verify the role of the *pheN* locus in a chromosomal location, the Tn*5 lacZ* within the insertion located in the *pheN* locus (pSISG5:: Tn*5lacZ*/ins-i) was introduced into the chromosome of wild-type (1116S) *P. tolaasii* by transplacement techniques to yield *P. tolaasii* 1116-MR1. The position of the substitution of the transposon-disrupted fragment for the wild-type fragment was confirmed by Southern blot analysis (results not shown). The location of Tn*5lacZ* in the *pheN* locus was also verified by PCR with primers against *pheN* sequences (results not shown). The characteristics of the resultant strain, 1116S-MR1, were found to conform to those of 1116R in all respects (Table 2). The reversion frequency of 1116S-MR1 to 1116S was checked in PB liquid medium and on PAF with or without kanamycin. It was found that the Tn*5lacZ* insertion into the *pheN* locus on the chromosome was stable, and in contrast to the spontaneous transition of 1116R to Phe^+, no reversion of 1116S-MR1 cells to Phe^+ was observed. Finally, introduction of pSISG29 or pSISG3 resulted in complementation of *P. tolaasii* 1116-MR1 from Phe^- to Phe^+ (results not shown). It was therefore deduced from these results that expression of the *pheN* locus is required for maintenance of the wild-type phenotype and that mutation of the *pheN* locus results in switching to the phenotypic variant form.

Analysis of the *pheN* nucleotide sequence. A 3,079-bp region of DNA spanning the *pheN* locus (Fig. 2) was sequenced and analyzed for the prediction of protein coding regions by the positional base frequency method (47) in all three possible reading frames. Translation of the preferred open reading frame (ORF) in the direction of transcription determined by Tn*5lacZ* mutagenesis (Fig. 3) employing a *Streptomyces* codon usage table demonstrated an ORF 2,727 nucleotides long, which could code for an 83-kDa protein 909 amino acid residues long (Fig. 3). The ORF was analyzed for codon bias pattern characteristics of organisms with a high G+C content, such as *Pseudomonas* spp. (4, 55). The G+C contents at codon positions 1, 2, and 3 were 71.3, 41.9, and 86.8%, respectively, indicating that the ORF identified in Fig. 3 is likely to be correct. The ORF extended more than 200 bases past the pSISG3 subclone, which is capable of reverting the phenotypic variant to Phe^+. This suggests that the pSISG3 subclone encodes a truncated but still functional protein. A likely Shine-Dalgarno sequence is indicated at positions 249 to 254 (AG GAG), adjacent to the predicted GTG translational start site at position 259 (Fig. 3). It has been shown that initial GTG codons specify *N*-formyl methionine if they are used as the first amino acid (17). This is a likely candidate for a ribosome-binding site because of its proximity to the first hydrophobic region described below. The nucleotide sequence upstream from the mRNA start site was analyzed for promoter sequences, but no such sequences that were easily recognizable as consensus sequences for either σ^{70}- or σ^{54}-type *Pseudomonas* promoters (13) were found. Those *Pseudomonas* promoters that are known to regulate virulence genes do not have any obvious conserved sequences among them and do not resemble other types of *Pseudomonas* promoters (13).

Comparison of the predicted amino acid sequence of the *pheN* gene with other known sequences. The *pheN* nucleotide sequence and predicted amino acid sequence were used to conduct a Fast A search of the nucleotide (EMBL) and protein sequence (PIR and Swiss-Protein) databases. Sequence similarities to the members of a class of environmentally responsive bacterial proteins known as two-component regulators were found. From this search, 87.5% similarity in a 908-amino-acid overlap to the predicted amino sequence of the *lemA* gene controlling multiple factors, including regulation of toxin and protease production in *Pseudomonas syringae* pv. syringae (22), was most noticeable (Fig. 4). In addition, similarities were found to segments of RcsC (26% similarity over a 544-amino-acid overlap [51]), ArcB (26% similarity over a 524-amino-acid overlap [27]), and PhoR (29% similarity over a 369-amino-acid overlap [30]) of *E. coli*; RpfC of *Xanthomonas campestris* (46% similarity over a 249-amino-acid overlap [52]); and BvgS of *Bordetella pertussis* (41% similarity over a 257-amino-acid overlap [2]). The sequence relatedness of PheN to RcsC and RpfC was confirmed through use of the RDF2 program (34). After 100 permutations, no random score approached the observed optimized score, indicating that the original alignment was biologically meaningful.

A diagrammatic comparison of the sequence of the predicted PheN protein with known sequences is shown in Fig. 4. Two probable transmembrane hydrophobic domains are present within the putative PheN amino acid sequence from nucleotides 288 to 357 and 762 to 828, containing 23 and 22 amino acids, respectively (Fig. 3). Comparison of the predicted amino acid sequences of PheN, LemA, RcsC, ArcB, BvgC, AlgB, and NtrC indicated that all of the individual residues that are conserved in most HPK-RR pairs are present in the proposed PheN amino acid sequence (Fig. 4 and 5). Several members of the two-component regulatory family have DNA-binding domains characterized by helix-turn-helix motifs (5) at their RR carboxy termini (21, 41). No such structure was identified in the PheN protein sequence by using the Genetics Computer Group program.

Hybridization of the cloned *pheN*-containing region of DNA to the wild-type and phenotypic variant genomic DNA. In order to determine whether there are differences in the *pheN* gene in 1116R compared with 1116S, total genomic DNA was restricted and analyzed by Southern hybridization with a *pheN* gene probe. The results (Fig. 6) demonstrate the presence of an additional 650-bp band in 1116R compared with 1116S. The 650-bp band is not seen in a Phe^+ revertant of 1116R. This observation suggests that a reversible DNA rearrangement occurs within the *pheN* locus during switching from 1116S to 1116R. The nature of this rearrangement is currently being elucidated.

Transcriptional regulation of *pheN* gene expression in *P. tolaasii*. In vitro expression of β-galactosidase by haploid *P. tolaasii* 1116S-MR1 (carrying a chromosomal *pheN*::*lacZ* transcriptional fusion) under different growth conditions was studied in full-strength and 1/10-strength PB medium in order to investigate the kinetics and effect of nutrient status on the expression of the *pheN* gene. A continuous increase in expression of the *pheN*::*lacZ* fusion during the logarithmic and stationary phases of growth was observed in PB liquid medium, 1/10-strength PB liquid medium, and in spent culture supernatant from wild-type *P. tolaasii* (Fig. 7). However, in 1/10-strength PB liquid medium and in spent culture supernatant, the rate of increase was significantly lower than in full-strength PB liquid medium, and an almost steady state after 30 h, corresponding to the stationary growth phase, was observed. These results suggest that depletion of nutrients in the growth medium directly or indirectly regulates expression of the *pheN* gene. Furthermore, the lower levels of *pheN*::*lacZ* expression in spent culture supernatant of wild-type *P. tolaasii* in comparison with 1/10-strength PB liquid medium could be due to the combined effects of nutrient deprivation and the synthesis of compounds (autoregulators) by bacteria themselves, which at

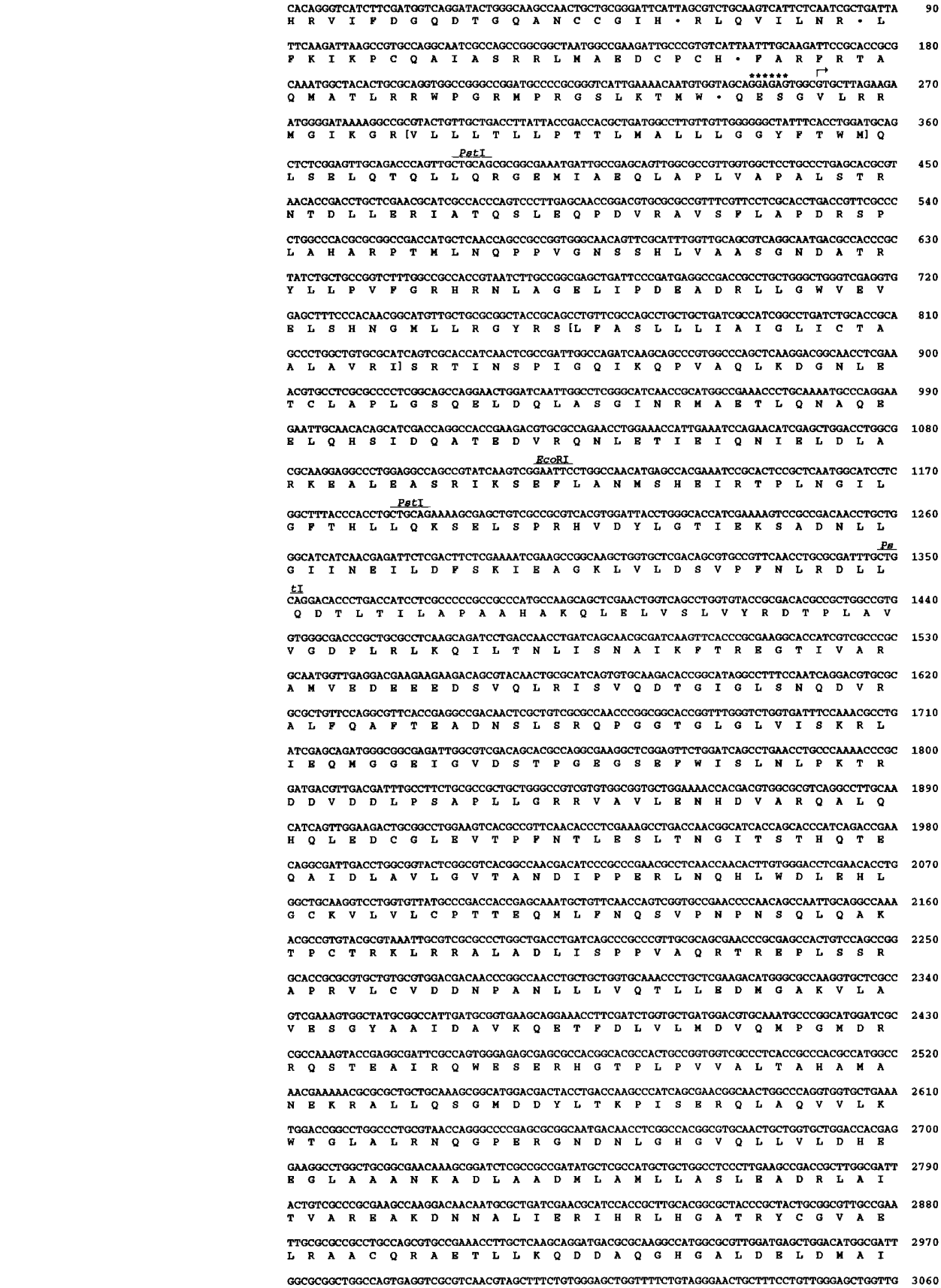

FIG. 3. Complete nucleotide and predicted amino acid sequence of the *pheN* ORF. Restriction sites are indicated by horizontal lines. The putative translational start codon is indicated by an arrow, and the most likely ribosome-binding site is indicated by asterisks. Bracketed amino acids are located in the two hydrophobic regions.

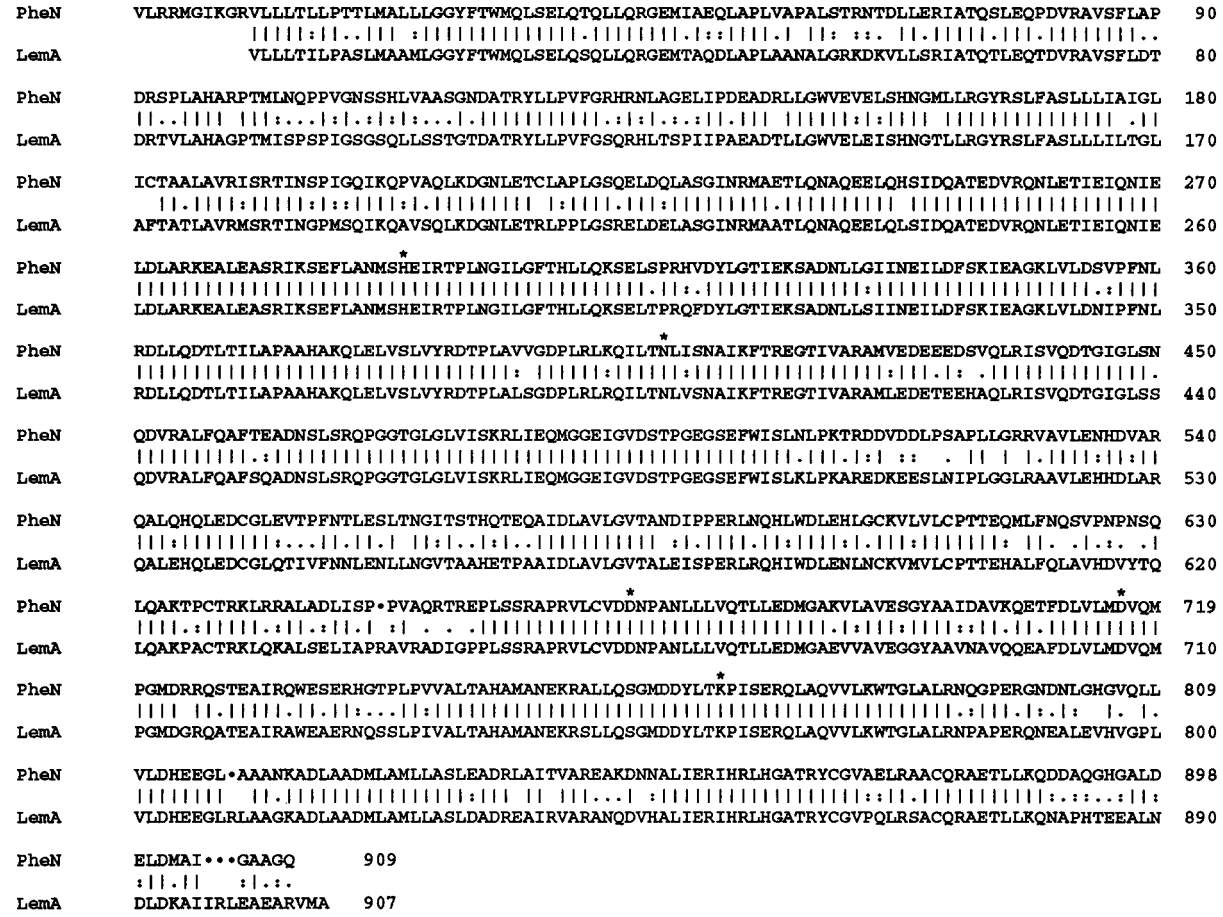

FIG. 4. Comparison of the putative amino acid sequences of PheN and LemA (22). Identical amino acids are connected by solid lines, amino acids which are conservative replacements are connected by two dots, and similar amino acids are connected by one dot. The putative PheN and LemA amino acid sequences show 66% identity and 87.5% similarity. The conserved histidine (H), asparagine (N), aspartic acid (D), and lysine (K) residues are marked with asterisks.

specific concentrations cause repression of the *pheN* gene. To determine whether the repression observed during growth on wild-type spent culture media was a general reflection of nutrient deprivation or was due to the cumulative effect of the poor nutrient status and compounds produced by wild-type *P. tolaasii*, the expression of *pheN*::*lacZ* by *P. tolaasii* 1116S-MR1 on a plate containing fresh PAF (20 ml) supplemented with ~5 ml of extract from PAF medium with 5-day-old wild-type *P. tolaasii* colonies was analyzed. The level of expression of β-galactosidase activity in plates containing PAF plus the extract from wild-type *P. tolaasii* culture media was significantly lower than that on the control plate containing PAF medium (results not shown). These observations suggest that *P. tolaasii* may produce autoregulatory compounds which regulate the expression of the *pheN* gene in a manner similar to that of *N*-(β-ketocaproyl)homoserine lactone in *Erwinia* spp. (3).

DISCUSSION

Wild-type *P. tolaasii* (1116S) colonies have previously been shown to produce a stable phenotypically distinct form called the phenotypic variant (1116R) at their margins. The process of transition from the wild type to the phenotypic variant form affects the ability of this pathogen to cause disease on its natural host, *A. bisporus* (10). Although these two forms of *P. tolaasii* differ from each other in a number of characteristics, the loss of ability of the phenotypic variant to cause brown blotch disease of *A. bisporus* is mainly attributed to its inability to synthesize the toxin tolaasin (6, 32). In previous studies (10, 57), unlike its equivalent wild-type form, the phenotypic variant form was reported to be stable and the transition from phenotypic variant to wild type was not generally observed in

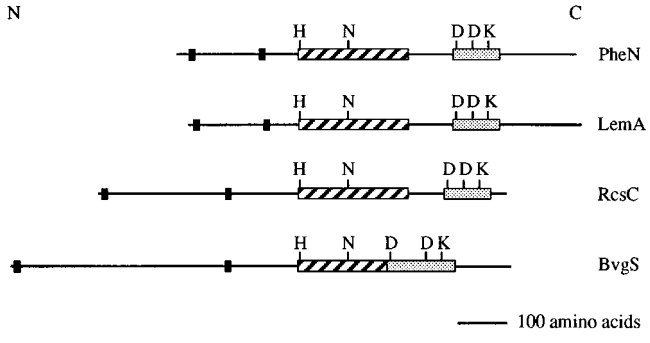

FIG. 5. Schematic alignment of the predicted *P. tolaasii* PheN protein with the *P. syringae* pv. syringae LemA, *E. coli* RcsC, and *B. pertussis* BvgS proteins. Small black squares, hydrophobic domains; hatched boxes, regions with homology to the conserved histidine protein kinase domain; stippled boxes, regions with homology to the conserved response regulator domain. Conserved amino acids are marked above the conserved histidine protein kinase and response regulator domains.

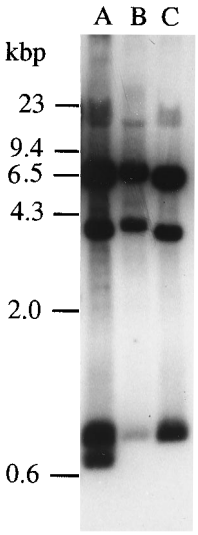

FIG. 6. Southern hybridization analysis of genomic DNA with a *pheN* probe. Genomic DNA from *P. tolaasii* 1116R (lane A), 1116S (lane B), and a Phe$^+$ revertant of 1116R (lane C) was digested with *Pst*I, separated by 0.7% agarose gel electrophoresis, and blotted and hybridized with a ^{32}P-labelled *Xho*I-*Bam*HI 3.4-kb probe corresponding to the *pheN* gene (Fig. 2e).

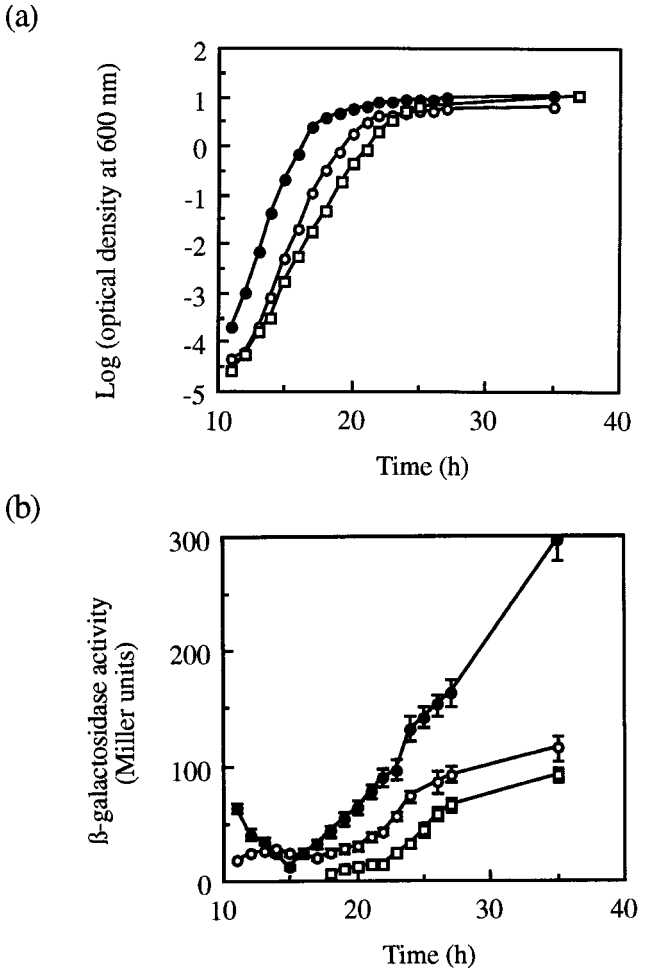

FIG. 7. Induction kinetics of *P. tolaasii* chromosomal *pheN*::*lacZ* fusion. *P. tolaasii* 1116S-MR1 was grown in PB liquid medium (solid circles), 1/10-strength PB liquid medium (open circles), or PB liquid medium supplemented with *P. tolaasii* wild-type culture filtrate (open squares) as described in Materials and Methods. Growth kinetics (a) and β-galactosidase activity (in Miller units) (b) in triplicate samples with standard errors are shown. The data are representative of results from at least three separate experiments.

culture. However, in the present study, it was found that the change from wild type to phenotypic variant is reversible, and reversion of 1116R to 1116S at a frequency of approximately 1 in 10^6 cells was observed. The appearance of a second unstable phenotypically distinct form distinguishable from 1116R around the margins of wild-type colonies which reverts to the wild-type form on subculture (data not shown) suggests that at least two different mechanisms regulate expression of phenotypic forms in *P. tolaasii*.

It has been shown here that a single locus, designated *pheN*, whose role appears central in the process of phenotypic variation, is responsible for altered regulation of the set of traits associated with phenotypic variation in *P. tolaasii*. The *pheN* ORF determined by sequencing is longer than the *pheN* region defined by subcloning and transposon mutagenesis (Fig. 2). A possible explanation for this discrepancy is that transposon insertions in the 3′ end of the gene may not completely inactivate the *pheN* gene product. In the case of pSISG3, only 30% of the transmerodiploids instead of 100% (as in the case of pSISG29) expressed wild-type behavior. The reason for the inability of pSISG3 to restore all 1116R cells to 1116S is not clear, but it may possibly be due to the instability of the cosmid or to pSISG3 carrying a truncated copy of the *pheN* locus, in which case higher levels of the *pheN* gene product may be required. The hypothesis that pSISG3 is capable of encoding a partially functional PheN protein is supported by the observation that all of the conserved domains of the PheN protein are located on the truncated protein encoded by pSISG3. It has been previously noted that the *lemA* gene, controlling the expression of multiple phenotypic traits in *P. syringae* pv. syringae, with Tn*3*::*HoHo1* in its 3′ end is functional (22). Similar loci which control the expression of multiple traits linked to the phenotypic variation have also been cloned from other plant-pathogenic bacteria, including *phcA* and *epsR* in *Pseudomonas solanacearum* (8, 24) and *rpfC* in *X. campestris* (52).

Comparison of the predicted amino acid sequence of the PheN protein with other known protein sequences demonstrated that it has a sequence similarity to members of a family of the two-component regulatory proteins (1, 21, 41). These include the LemA (22), BvgS (2), RcsC (51), PhoR (30), ArcB (27), and RpfC (52) proteins, which coordinately regulate the expression of multiple genes in response to environmental conditions. An interesting feature of these two-component regulators is that the HPK and RR domains normally found on different proteins (50) are located on a single peptide. The predicted amino acid sequence of the PheN protein thus contains all of the important conserved residues of both the HPK and RR domains, which confirms the regulatory nature of the *pheN* gene product. Alignment of these proteins illustrated the characteristic conserved spacing between the residues in the HPK domains as well as between those in the RR domains (Fig. 5). The high degree of sequence homology between PheN and other two-component transcriptional regulators such as LemA suggests that the PheN protein functions by a similar mechanism and controls the expression of other genes at the transcriptional level. However, it is not clear whether the *pheN* gene product directly modulates the expression of other genes or whether other gene products are also required for this process. The lack of a helix-turn-helix motif in the PheN receiver domain makes a direct role in target promoter recogni-

tion unlikely. Two hydrophobic domains in the amino terminus of the PheN protein, similar to those of BvgS, RcsC, and other sensor proteins which have some of their portion in the periplasmic space, were observed. It is therefore probable that like other sensor proteins, the PheN protein assumes a similar configuration to sense external environmental signals with its extracytoplasmic domain. Given the DNA polymorphism seen in the *pheN* gene of 1116R, it is tempting to speculate that mutations in *pheN* and its coordinated regulation, as seen in the *bvgS* locus of *B. pertussis* (49) and *phcA* of *P.* (*Burkholderia*) *solanacearum* (7) may be involved in the metastable reversible transition from one cell type to another.

Preliminary experiments carried out to identify factors regulating expression of the *pheN* gene suggested that phenotypic variation in *P. tolaasii* is a social phenomenon, which occurs when cells are present at high density. The appearance of white sectors indicating repression of *pheN*::*lacZ* expression in areas of confluent growth and cell density-dependent expression of the *pheN* gene implies that the bacteria are responding to the presence of other cells and probably to the presence of substances produced by other cells. However, depletion of nutrients because of high cell density can be an important factor. The observed effects of culture extracts on *pheN* expression support the hypothesis that autoregulators produced by the wild-type *P. tolaasii* regulate expression of the *pheN* gene.

On the basis of our current knowledge, the following model is proposed for PheN location and function. The two hydrophobic domains anchor PheN in the cytoplasmic membrane, with its carboxy-terminal transmitter and regulator domains located in the cytoplasm. The amino-terminal extracytoplasmic domain of the PheN protein may sense the environment and transfer signals to its carboxy-terminus response regulator module via the transmitter domain. The response regulator domain then controls the expression of other genes either directly or via a second unknown component. This phenotypic switch appears to be regulated at three different levels. First, in the presence of stimulatory environmental signals, PheN activates either directly or indirectly the expression of the genes involved in tolaasin synthesis, protease production, and mucus production but represses certain other genes controlling siderophore production, motility, chemotaxis, etc. Second, the presence of autoregulatory compounds at a threshold concentration and poor nutrient conditions might completely eliminate expression of the *pheN* gene itself and result in the appearance of the highly unstable phenotypic variant form which appears around the margins of wild-type colonies and reverts back to the wild-type form when subcultured onto fresh medium. Third, the appearance of the metastable phenotypic variant form, which arises as sectors at the margins of wild-type colonies, is a result of DNA rearrangement associated with the *pheN* locus. Experiments are currently in progress to further analyze these effects.

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