rtpA, a gene encoding a bacterial two-component sensor kinase, determines pathogenic traits of *Pseudomonas tolaasii*, the causal agent of brown blotch disease of a cultivated mushroom, *Pleurotus ostreatus*

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Pseudomonas tolaasii strain PT814 produces extracellular toxins, tolaasins, and a volatile toxin, tovsin, that are responsible for the induction of brown blotch and rotting, respectively, in a cultivated mushroom, *Pleurotus ostreatus*. Insertions of single transposon mini-Tn5km1 into the chromosome of *P. tolaasii* strain PT814 generated mutants that are pleiotropically defective in tolaasin and protease production, and altered in colony morphology. The mutants, however, produce tovsin at the level of wild-type. Variants phenotypically similar to the pleiotropic mutants of *P. tolaasii* strain PT814 spontaneously occurred in *P. tolaasii* strain S8501 at 22–30°C in vitro. The occurrence of variants was significantly reduced in the presence of extracts of *P. ostreatus* or at a temperature of 15–20°C. The *rtpA* gene (*rtpA* = regulator gene of tolaasin production and other pleiotropic traits) isolated from a *P. tolaasii* strain PT814 gene library restored the wild-type phenotype in both the mini-Tn5km1 insertion and spontaneous mutants. mini-Tn5km1 insertions were also located in the allele of *rtpA*. Nucleotide sequencing of the *rtpA* DNA revealed an open reading frame of 2,751 bp predicted to encode a protein consisting of 917 amino acid residues with a molecular mass of 100.6 kDa and displaying the conserved amino acid sequence of both sensor and receiver domains of "bacterial two-component regulators." The data suggest that the machinery responding to environmental stimuli is essential for the pathogenic interaction of *P. tolaasii* with the mushroom.

Key Words——*Pleurotus ostreatus*; *Pseudomonas tolaasii*; *rtpA*; two-component regulator.

Pseudomonas tolaasii Paine is the causal agent of brown blotch disease in cultivated mushrooms, *Pleurotus ostreatus* (Jacq.: Fr.) Kummer and *Agaricus bisporus* (Lange) Singer (Suyama and Fujii, 1993; Tolaas, 1915). The primary determinant of pathogenicity is extracellular toxins, i.e., tolaasins, produced by the bacterium (Murata and Magae, 1996; Nutkins et al., 1991; Rainey et al., 1993; Shirata et al., 1995). Chemical and genetic analyses of *P. tolaasii* strain NCPPB1116, an isolate from diseased *A. bisporus*, first revealed the identity of tolaasin as a low-molecular weight lipodepsipeptide, which is apparently synthesized by the products encoded by a ca. 65 kb DNA segment of the bacterium (Nutkins et al., 1991; Rainey et al., 1993). Tolaasin induces the disease by damaging the host cells through its action on cell membranes as a biosurfactant (Hutchison and Johnstone, 1993).

Recent developments in the molecular approach to plant/mammal-bacterium interactions have revealed that the expression of a pathogenic process in a bacterium is controlled by signals from the hosts and regulatory elements in the bacterium that respond to such stimuli (Alfano and Collmer, 1996; Dziejman and Mekalanos, 1995). A typical case is the bacterial two-component regulatory system (Stock et al., 1989; Stock et al., 1995). Among the regulators belonging to this class, *bvgS/lemA* and *gacA* families have been identified in fluorescent pseudomonads that control the production of various antifungal agents (Corbell and Loper, 1995; Hrabak and Willis, 1992; Laville et al., 1992). Regulators belonging to the *bvgS/lemA* family include sensor histidine protein kinase represented by the *bvgS* gene product of *Bordetella pertussis* (Bergey, Harrison, Breed, Hammer et Huntoon) Moreno-Lopez, in which signals are transmitted by a series of phosphorylation relays to the response regulator (Uhl and Miller, 1995). By contrast, GacA is a response regulator which receives signals by phosphorylation and activates the expression of the target gene (Laville et al., 1992). The *pheN* gene of *P. tolaasii* strain NCPPB1116 that is required for tolaasin production is reported to be a regulator gene belonging to

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the *bvgS/lemA* family (Grewal et al., 1995).

While the pathogenic mechanisms of a *P. tolaasii* isolate from *A. bisporus* in Europe have been intensively studied (Grewal et al., 1995; Hutchison and Johnstone, 1993; Nutkins et al., 1991; Rainey et al., 1993), little is known about the interaction between *P. tolaasii* and *P. ostreatus*. Analysis of genes involved in the host-parasite relationship between *P. tolaasii* and *P. ostreatus* is of interest primarily in view of the following two aspects. (i) It has been reported that there is a great diversity among *P. tolaasii* isolates (Thorn and Tsuneda, 1996), suggesting their unique relationship with ecologically different hosts, e.g., *A. bisporus*, a meadow mushroom, versus *P. ostreatus*, a wood-rotting fungus (Chang and Miles, 1989). (ii) *P. tolaasii* isolated from *P. ostreatus* has been demonstrated to show interesting behavior in parasitism involving patho-toxins under genetic control (see below).

Brown blotch disease in *P. ostreatus* caused by the bacterium was first reported by Suyama and Fujii in 1993, many years after the discovery of a similar disease in *A. bisporus*. The recognition of the disease, however, contributed much to the development of cultivation worldwide of specialty mushrooms including *P. ostreatus*. Subsequently, tolaasins of *P. tolaasii* strains isolated from diseased *P. ostreatus*, PT814 and S8501, were identified as elicitors of pathogenesis and found to be chemically similar to tolaasins reported in a diseased *A. bisporus* isolate, NCPPB1116 (Murata and Magae, 1996; Shirata et al., 1995). In addition to general similarities, however, unique features of the interaction between *P. tolaasii* and *P. ostreatus* were recognized. For example, *P. ostreatus* developing the brown blotch symptom continues to rot due to a volatile toxin, tovsin, produced by *P. tolaasii* only when it is in contact with *P. ostreatus* (Shirata, 1996). Preliminary data also indicate that components of *P. ostreatus* fruiting bodies activate tolaasin production (Murata and Magae, 1996), supporting the hypothesis that *P. tolaasii* strains causing disease in *P. ostreatus* have a machinery enabling them to interact specifically with *P. ostreatus*.

In the present study, we attempted to analyze the genetic control of pathogenicity of *P. tolaasii* isolated from diseased *P. ostreatus* in Japan. Two-types of avirulent mutants of *P. tolaasii* were characterized: one generated by using a transposon delivery system, *Escherichia coli* (Migula) Castellani et Chalmers strain S17-1*λpir* (pUT::mini-Tn5km1) (De Lorenzo et al., 1990), on a genetically stable *P. tolaasii* strain PT814; and the other that spontaneously occurs at a high rate in response to changes in growth conditions. The study demonstrates that a gene encoding the *bvgS/lemA* family of the bacterial two-component regulator is required for the induction of brown blotch disease in *P. ostreatus*, the regulator being similar to but not identical with *pheN* of *P. tolaasii* strain NCPPB1116. Together with our previous observations on the activation of pathogenicity factors by host components, the present analysis provides a basis for future investigations into the signal transduction system functioning during the interaction between *P.*

tolaasii and *P. ostreatus*.

Materials and Methods

Bacterial strains and plasmids Bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* and *P. tolaasii* strains carrying drug markers were maintained on Luria-Bertani (LB) agar and Pseudomonas agar F (PAF), respectively, supplemented with antibiotics as required.

Source of cultivated mushrooms *Pleurotus ostreatus* cultivated in a spawn containing sawdust (Chang and Miles, 1989) was provided by a local mushroom grower.

Media LB, PAF, and its liquid version (PF-broth) were prepared as recommended by the manufacturer (Difco). Standard compositions of a minimal salts-carbohydrate medium and water extracts of fruiting bodies of *P. ostreatus* (OME; oyster mushroom extracts) were described previously (Murata and Magae, 1996; Murata et al., 1991). Decoction of cultivated mushrooms was prepared in the same manner as for water extracts except that water containing the fruiting bodies was boiled for 30 min instead of being incubated at room temperature for 2 h. When desired, antibiotics were added as follows (μg/ml): ampicillin (Ap), 50; chloramphenicol (Cm), 50; kanamycin (Km), 50 for *E. coli* and 100 for *P. tolaasii*; tetracycline (Tc), 10 for *E. coli* and 100 for *P. tolaasii*. Media were solidified by the addition of 1.5% agar (Wako Pure Chemical, Osaka).

Toxin assay The production of tolaasin was examined by observing the occurrence of a white line (WL) on PAF (Rainey et al., 1993; Wong and Preece, 1979), or blackening of a potato tuber slice (Shirata et al., 1995). The levels of tolaasin were semiquantitatively determined by scoring the extent of the zone of inhibition of *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis, Gillaspie, Vidaver et Harris on PAF after the application of serially diluted tolaasin samples (Murata and Magae, 1996; Shirata et al., 1995).

The level of tovsin was determined by the inhibition of germination of *Kactuca sativa* L. seedlings as described by Shirata (1996). Briefly, *Kactuca sativa* seeds on a wet filter disk and *P. ostreatus* fruiting bodies inoculated with *P. tolaasii* were placed in separate petri dishes without lids, which were then incubated together in a sealed container at 20°C for several days. Inocula which inhibited the germination of *Kactuca sativa* seedlings were considered to be tovsin-producing strains.

Extracellular protease assay Nutrient gelatin agar was used to determine the extracellular protease activity (Murata et al., 1994). For quantitation, bacteria were cultivated in PF-broth in a 24°C rotary shaker to the stationary growth phase, culture supernatants were obtained by centrifugation (4°C, 12,000×*g*), filter-sterilized (pore size=0.45 μm; Advantech, Tokyo), serially diluted, and applied in an appropriate volume on a 3% gelatin agar plate, and the extent of the halo formed was scored.

mini-Tn5km1 insertion mutagenesis in *P. tolaasii* strain PT814 The mini-Tn5km1 was introduced into *P. tolaasii*

Table 1. Bacterial strains and plasmids.

Bacteria/plasmids	Relevant characteristics	References/sources
Bacterial strains		
Pseudomonas tolaasii		
PT814	Wild type, Ap^r Cm^r	Suyama and Fujii (1993)
S8501	Wild type, Ap^r Cm^r	Shirata et al. (1995)
MUR31, MUR33, MUR34, MUR35	*rtpA*::mini-Tn5km1, Tox$^-$ Path$^-$ Prt$^-$ Col$^-$, Ap^r Cm^r Km^r	Derived from PT814 by mini-Tn5km1 insertion mutagenesis (this study)
S8501C	Tox$^-$ Path$^-$ Prt$^-$ Col$^-$, Ap^r Cm^r	Phenotypic variants of S8501 (this study)
Escherichia coli		
HB101	*pro hsdR hsdM recA*, Sm^r	Takara Shuzo
Jm109	*recA1 thi lacI^q* Δ*(lacZ)M15* Δ*(lac-proAB) endA1 hsdR17(r$_K^-$ m$_K^+$)*	Takara Shuzo
S17-1λ*pir*	C600::RP4-2 Tc::Mu-Km::Tn7 *pro hsdR hsdM$^+$ recA pir$^+$*	De Lorenzo et al. (1990)
Plasmids		
pBleuscript SK$^+$	Ap^r	Stratagene
pCR2.1	Ap^r Km^r	Invitrogen
pLAFR5	Mob$^+$ Tc$^+$	Keen et al. (1988)
pRK415	Mob$^+$ Tc$^+$	Keen et al. (1988)
pRK2013	Mob$^+$ Tra$^+$, Km^r	Figuriski and Helinski (1979)
pUT::mini-Tn5km1	Mob$^+$, Km^r Ap^r	De Lorenzo et al. (1990)
pHHM125	*rtpA$^+$*, Tc^r	pLAFR5 containing PT814 genomic DNA (this study)
pHHM134	*rtpA$^+$*, Tc^r	pRK415 containing a 7.4 kb DNA segment of pHHM125 (this study)
pHHM135	*rtpA$^+$*, Ap^r	pBluescript SK$^+$ containing a 4.5 kb *Bg*lI-*Eco*RI fragment of pHHM134 (this study)
pHHM136	*rtpA$^+$*, Tc^r	pRK415 containing a 4.5 kb *Bg*lI-*Eco*RI fragment of pHHM134 (this study)

strain PT814 by mating with *E. coli* strain S17-1λ*pir* carrying the pUT::mini-Tn5km1 as described by De Lorenzo et al. (1990). Transconjugants selected on PAF containing Km and Cm were inoculated to the fruiting bodies of *P. ostreatus* by using sterile toothpicks and the inoculated specimens were incubated in a moist chamber at 20°C for 48 h. The extent of development of disease symptoms was visually determined.

Pathogenicity test Bacteria from overnight cultures on minimal salts-glucose agar were suspended in sterile water. The bacterial suspension containing ca. 2.0×10^6 cells was inoculated by using a micropipet onto the top surface of *P. ostreatus* fruiting bodies. Mushrooms were placed in a moist chamber at 20°C for 48 h and the extent of brown blotch development was recorded.

Recombinant DNA techniques Standard procedures were used for the isolation of plasmid and chromosomal DNA, transformation of *E. coli*, restriction digests, gel electrophoresis, electroelution of DNA fragments, and DNA ligations (Sambrook et al., 1989). After hybridization, high-stringency washing of the blots was performed as previously described (Murata et al., 1990). Restriction and modifying enzymes were obtained from New England Biolab (Beverly, MA) and Takara Shuzo (Otsu), respectively.

Localization of mini-Tn5km1 in genomic digests of

mutant DNA Southern hybridization analysis was conducted to determine the mini-Tn5km1 insertion sites. Genomic DNA of the mutants and of the parent strain was digested with one of the restriction endonucleases, *Cla*, *Eco*RI, *Kpn*, *Sac*I, and *Xho*I, that asymmetrically cut once within mini-Tn5km1, and probed with the 1.8 kb *Eco*RI fragment of pUT::mini-Tn5km1 that contains most of mini-Tn5km1 and the cloning site of pUT vector (De Lorenzo et al., 1990) and with the cloned 6.5 kb *Kpn*-*Sac*I *rtpA* DNA (see below).

Isolation of *rtpA* cosmids A standard protocol was used for constructing a gene library of *P. tolaasii* strain PT814 in a cosmid, pLAFR5, (Keen et al., 1988). Individual cosmids were transferred by using *E. coli* strain HB101 carrying pRK2013 as the mobilizing system (Figurski and Helinski, 1979) into representative mini-Tn5km1 insertion mutants. The method described by Murata et al. (1990) was used to conduct triparental mating. Transconjugants selected on PAF containing Cm and Tc were tested for the restoration of tolaasin and protease production by potato tuber slice and nutrient gelatin agar assays, respectively.

Unidirectional deletion to locate *rtpA* Unidirectional deletion was implemented on the insert DNA of pHHM135 from both ends by using TaKaRa Kilo Deletion Kit (Takara Shuzo, Otsu). Resulting constructs were selected to

locate *rtpA* by allowing complementation with RtpA⁻ mutants. Before complementation analysis, insert DNA of deletion derivatives was amplified by using TaKaRa LA PCR Kit and M13–21/M13 reverse primers (Takara Shuzo, Otsu). The products were ligated into pCR2.1 (Invitrogen, San Diego, CA), excised by using appropriate restriction enzymes to only cut the multiple cloning site of the vector, ligated into pRK415, and introduced into RtpA⁻ mutants by triparental mating as described above. The direction of insert DNA in pRK415 was manipulated by double digests of the pCR2.1 constructs such as *Xba*I-*Hind*III and *Xba*I-*Sac*I and subsequent cloning at the same restriction sites in pRK415. This lengthy procedure was required because the only drug marker available to maintain plasmids in *P. tolaasii* strain PT814 and its derivatives was Tcʳ, and the insert DNA of the deletion constructs could not be excised for subsequent cloning due to the lack of restriction sites after the unidirectional deletion process. The nucleotide sequence of ca. 600 bp at both ends of the PCR products was analyzed prior to the transcloning into pRK415 by using Dye Primer Cycle Sequencing FS Core Kit with M13-21/M13-reverse primers (Perkin Elmer Japan) and ABI 377 Prism (Perkin Elmer Japan) to confirm that the products were the expected ones.

Nucleotide sequence of *rtpA*   Overlapping deletions in both strands differing in size by ca. 0.3–0.5 kb described above were sequenced by using Dye Primer Cycle Sequencing FS Core Kit (Perkin Elmer Japan) and ABI 377 Prism (Perkin Elmer Japan). Data were analyzed by the GENETYX program ver. 8 (Software Development, Tokyo). Advanced BLAST Search provided by the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov/BLAST) was used for homology search.

Nucleotide sequence accession number   The sequence of the *rtpA* gene has been assigned the DDBJ accession number AB002529.

Results

Isolation of pleiotropic avirulent mutants of *P. tolaasii* strain PT814   Of the 2500 Kmʳ colonies resulting from the mating with *E. coli* strain S17-1λpir (pUT::mini-Tn5km1), four colonies pleiotropically failed to induce brown blotch in *P. ostreatus* fruiting bodies (Path⁻), to produce tolaasin (Tox⁻) and extracellular protease (Prt⁻), and to display normal colony morphology (Col⁻) on PAF containing 0.6% agar (Fig. 1). The production of tovsin, however, was not affected by the mutation (Fig. 2). Genomic digests of the mutants were analyzed by Southern hybridization with a probe of the 1.8 kb *Eco*RI DNA segment containing mini-Tn5 km1 to determine whether the pleiotropic phenotype is due to the insertion at a single gene locus (Fig. 3). The *Cla*I digests in *P. tolaasii* strain MUR31 showed two signals due to the site within the transposon, whereas digests with *Eco*RI, *Kpn*I, or *Sac*I produced one signal due to the site located near the end of the transposon (Figs. 3, 4). The three other strains, MUR33, MUR34, and MUR35, contained a single mini-Tn5km1 insertion at gene loci identical with that in
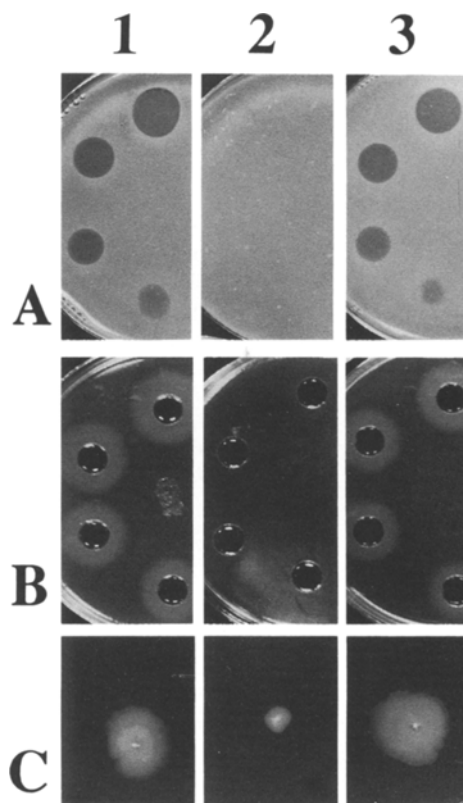
Fig. 1.   Phenotype of *Pseudomonas tolaasii* strain MUR31, an *rtpA*::mini-Tn5km1 mutant of *P. tolaasii* strain PT814, and the strain carrying an *rtpA* plasmid, pHHM125.
Levels of tolaasins (Tox: Panel A) and protease (Prt: Panel B) were determined in the supernatants of stationary growth phase cultures grown in minimal salts-glycerol medium containing water extracts of *P. ostreatus* fruiting bodies and PF-broth, respectively. Undiluted samples were spotted at the top of each plate, and samples with dilutions of 1/2, 1/4 and 1/8 were placed counter clockwise. The colony morphology (Col) was determined by stub-inoculation of bacteria in PAF containing 0.6% agar (Panel C). Columns 1, 2 and 3 show the phenotype of *P. tolaasii* strains PT814 (pLAFR5), MUR31 (pLAFR5) and MUR31 (pHHM125), respectively. For details of assay conditions for Tox, Prt and Col, see Materials and Methods.

MUR31. Data indicate that the pleiotropic phenotype was conferred by the insertion at a single gene locus, which is most likely a positive regulator gene. The phenotype was designated as RtpA (standing for mutation in a *r*egulator gene of *t*olaasin production and other *p*leiotropic traits).

Isolation of variants from *P. tolaasii* strain S8501   *Pseudomonas tolaasii* strain S8501 spontaneously produces variants phenotypically similar to the pleiotropic mutants described above in PF-broth at 22–30°C, whereas no segregants were found in *P. tolaasii* strain PT814 (Fig. 5). At 17 and 20°C, however, the occurrence of variants was markedly reduced, and a sizable portion of the bacterial cells remained viable even 20 d after the inoculation (Fig. 5). The colonies of the vari-
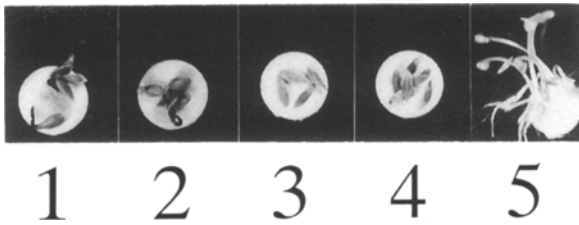
Fig. 2. Production of volatile toxin, tovsin, in *Pseudomonas tolaasii*.

Tovsin production was determined as described in Materials and Methods. The figure shows *Kactuca sativa* seeds coincubated with: 1, *P. tolaasii* strain S8501 (RtpA+); 2, *P. tolaasii* strain S8501C (RtpA−); 3, *P. tolaasii* strain PT814 (RtpA+); 4, *P. tolaasii* strain MUR31 (RtpA−); 5, buffer control.

Fig. 4. Restriction endonuclease map of the 6.5 kb *Kpn*I-*Sac*I *rtpA* DNA, and schematic representation of mini-Tn5km1 insertions identified in the mutant genome and unidirectional deletions implemented in pHHM135 to localize *rtpA*.

The open flags indicate mini-Tn5km1 insertion sites in RtpA− mutants of *P. tolaasii* strain PT814, MUR31, MUR33, MUR34, and MUR35. The thick line and the thin lines underneath depict the 6.5 kb *Kpn*I-*Sac*I *rtpA* DNA and the insert DNA of pHHM136 (stretching from *Bgl*II to *Eco*RI) as well as deletion derivatives of pHHM135 (marked B63, B52, B1, B44, E9, E1 and E5), respectively. The location and the predicted direction of *rtpA* transcription are indicated by the hatched bar and the arrow, respectively. Effect of subclones and deletion derivatives on the restoration of Tox+ Prt+ phenotype in *P. tolaasii* strains MUR31, MUR33, MUR34, MUR35, and S8501C is indicated in the right column. Restriction map of mini-Tn5km1 out-of-scale is boxed in. The broken line in 1.8 kb mini-Tn5km1 is a portion of the multiple cloning site in the pUT vector (De Lorenzo et al., 1990). Abbreviations: B, *Bgl*II; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; S, *Sac*I; Sa, *Sal*I; X, *Xba*I.
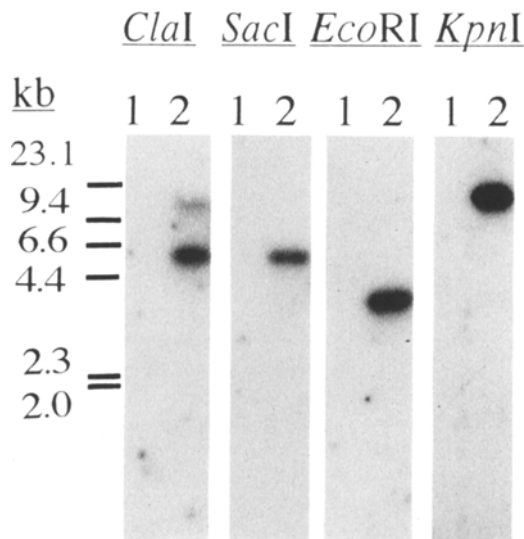

Fig. 3. Southern hybridization analysis of a mini-Tn5km1 insertion mutant, *Pseudomonas tolaasii* strain MUR31.

The 1.8 kb *Eco*RI fragment of pUT::mini-Tn5km1 (De Lorenzo et al., 1990) labeled with [32P]dCTP was used as a probe. Molecular size is shown at left. Single mini-Tn5km1 insertion in the pleiotropic mutants of *P. tolaasii* strain PT814 was confirmed by the presence of two hybridization signals in samples digested with *Cla*I, of which the restriction site is within the transposon (Fig. 4). In genomic digests with *Sac*I, *Eco*RI and *Kpn*I, an intense single band was observed in association with the sites located near one end of the transposon (Fig. 4). Lane 1, *P. tolaasii* strain PT814; Lane 2, *P. tolaasii* strain MUR31.

ants are translucent, mucoid, and fluorescent as compared to the opaque, rough, and slightly fluorescent colonies of the parent, *P. tolaasii* strain S8501. Ingredients of culture media also affected the rate of variant formation. When the decoction or the water extracts of *P. ostreatus* fruiting bodies was used as a medium, the maximum number of variants and variant formation were significantly reduced, compared to cultures grown in PF-broth (Fig. 6).

Molecular cloning of the *rtpA* gene A library of *P. tolaasii* strain PT814 containing about 2000 cosmid clones was mobilized into RtpA− strains, MUR31 and S8501C, and those which restored the Tox+ Prt+ phenotype were scored. We obtained four plasmids that restored the Tox+ Prt+ Path+ Col+ phenotype in the pleiotropic mutants (Fig. 1). These cosmids also complemented mutants MUR32, MUR33, and MUR34. Since restriction analysis revealed that the four RtpA+ cosmids consisted of the same DNA segment, we further investigated one representative plasmid, pHHM125, in detail.

The *rtpA* DNA segment of pHHM125 was subcloned by preparing *Sau*3A partial digests and ligating the fragments to *Bam*HI-cut pRK415 treated with phosphatase. The Tcr JM109 transformants showing white colonies in the presence of X-gal and IPTG were selected to be mobilized into the RtpA− mutants for testing for complementation. A plasmid, pHHM134, containing a 7.4 kb insert DNA of pHHM125 restored the RtpA+ phenotype in MUR31, MUR33, MUR34, MUR35, and S8501C and was used for further subcloning into pRK415 by utilizing
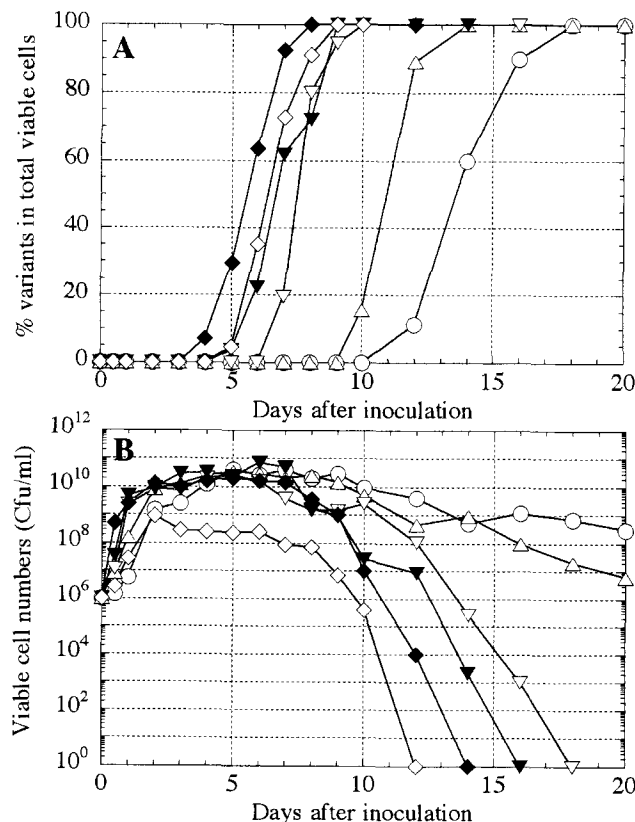
Fig. 5. Effect of growth temperature on viability and induction of phenotypic variants in *Pseudomonas tolaasii* strain S8501.
The bacterium was grown in PF-broth. At intervals, portions of cultures were harvested, serially diluted, and spread on PAF. Randomly picked variants were checked for defects in tolaasin and protease production by agar plate assays. No variants from *P. tolaasii* strain PT814 were detected under the experimental conditions. Panel A: Percentage of variants in total viable cells was determined by scoring the number of colonies that are translucent, mucoid, fluorescent, and defective in tolaasin and protease production against total viable cell number. Panel B: Viable cell number was expressed as colony forming units per ml of culture. Symbols: open circle, 17°C; open triangle directed upward, 20°C; open triangle directed downward, 22°C; closed triangle, 24°C; closed diamond, 27°C; open diamond, 30°C.

restriction sites in the fragment. The 4.5 kb *Bg*/II-*Eco*RI fragment located within the 6.5 *Kpn*I-*Sac*I DNA of pHHM134 was cloned into pRK415, generating pHHM136. This plasmid complemented all the mutants (Fig. 4). In addition, Southern hybridization analysis with probes, the *Kpn*I-*Sac*I *rtpA* DNA and the *Eco*RI DNA containing mini-Tn*5*km1, confirmed that the single mini-Tn*5*km1 insertion was located on the allele of *rtpA* in all the insertion mutants (Fig. 4).

Localization of the *rtpA* gene To locate *rtpA*, unidirectional deletion was implemented on pHHM135, pBluescript SK+ containing the 4.5 *Bg*/II-*Eco*RI fragment (see Materials and Methods). Following the deletion and the PCR amplification of the deleted inserts, the products were cloned into pRK415 in both directions downstream of the *lac* promoter (p*lac*) at the multiple cloning site of the vector (Fig. 4). In either orientation, the deletion derivatives designated as B63, B52, B1, and E9 were able to some extent to confer the RtpA+ phenotype to all the *rtpA*::mini-Tn*5*km1 mutants, while B44, E1, and E5 were not (Fig. 4). Deletion derivatives B52 and B1

showed different degrees of complementation depending upon the orientation of the cloned fragment. The fragments cloned under p*lac* from left to right as shown in Fig. 4 conferred the Tox+ Prt+ phenotype to all the *rtpA*::mini-Tn*5*km1 mutants at a level significantly higher than the fragments cloned in the opposite direction. This phenomenon is probably due to the driving of *rtpA* transcription by both the native promoter and p*lac* of the vector in the direction where the Tox+ Prt+ phenotype was more pronounced but by only the native promoter in the other direction. Deletion analysis along with the generation of the pleiotropic mutants carrying *rtpA*::mini-Tn*5*km1 strongly suggests that the *rtpA* gene is present in a ca. 3.5 kb DNA segment lying between *Bg*/II and the *Sal*I at right, and the direction of *rtpA* transcription is from left to right, as shown in Fig. 4.

Nucleotide sequence of *rtpA* By sequencing deletion derivatives of pHHM135, an open reading frame (ORF) was identified that corresponds to the range of *rtpA* and to the predicted direction of transcription as determined by the deletion analysis described above (Fig. 7). The
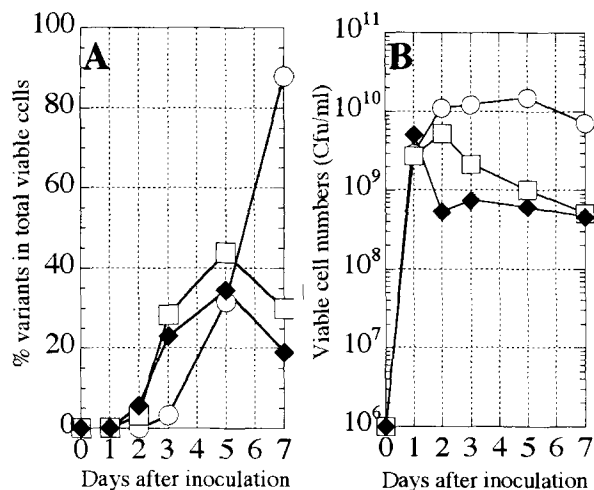
Fig. 6. Occurrence of phenotypic variants in *Pseudomonas tolaasii* strain S8501.
Bacteria were inoculated at a rate of ca. 1.6×10^6 cells/ml in a medium and incubated on a rotary shaker at 27°C for 7 d. Samples were removed from cultures at intervals, serially diluted, and spread on PAF to determine the number of viable cells and phenotypic variants. Colonies that were translucent, mucoid, and intensely fluorescent were considered to be variants. Randomly picked variants were checked for defects in tolaasin and protease production by agar plate assays. Panel A: Number of variant colonies × 100/total number of viable cells. Panel B: Total viable cell numbers expressed as colony-forming units per ml of culture. Symbols: open circle, PF-broth; open box, decoction of *P. ostreatus* fruiting bodies; closed box, water extracts of *P. ostreatus* fruiting bodies.

2,751 bp ORF is preceded by a putative Shine-Dalgarno sequence (AGGAGA; Shine and Dalgarno, 1974) located 6 bp upstream of the putative GTG start codon (Fig. 7). No other translational start codon is properly associated with a putative Shine-Dalgarno sequence. Also, the probability that a coding region is present in this ORF has a TESTCODE indicator of 1.31 as computed by the method of Fickett (1982). This value not only satisfies the standard of coding sequences (>0.95) but also gives one of the highest probabilities in the sequence of 4,159 bp DNA.

The RtpA protein is predicted to consist of 917 amino acid residues having a molecular mass of 100.6 kDa. It has an amino acid sequence strikingly similar to PheN of *P. tolaasii* strain NCPPB1116 and ApdA of *Pseudomonas fluorescens* (Trevisan) Migula strain Pf5, members of the BvgS/LemA family of bacterial two-component regulators (Figs. 7, 8). Two putative transmembrane hydrophobic domains of RtpA have a common amino acid sequence which is 96% and 84% identical with those of PheN and of ApdA, respectively. The predicted sensor kinase domain contains an amino acid sequence 98% identical with the *bvgS/lemA* family of regulators, and H, N, D/F and G boxes of most conserved residues among members of the histidine protein kinase superfamily were found in that order (Fig. 8; Stock et al., 1995). The

receiver domain was also predicted based on the presence of two asparatic acid residues, a threonine residue, and a lysine residue properly located so that it could function as the active site for phosphorylation (Fig. 8; Volz, 1995). The amino acid residues at putative active sites along with those involved in functional conformation seemed to be highly conserved in RtpA, as noted in PheN and ApdA. The lack of the helix-turn-helix DNA binding motif and the presence of the conserved C-terminal histidine region in the predicted RtpA product are characteristic of the regulators belonging to the *bvgS/lemA* family.

Discussion

The pathogenicity of *P. tolaasii* strains originally isolated from brown blotch disease of *P. ostreatus* in Japan was investigated genetically. Single mini-Tn5km1 insertion in *P. tolaasii* strain PT814 gave rise to mutants that were unable to produce tolaasins (Tox⁻) and extracellular proteases (Prt⁻), were not pathogenic to *P. ostreatus* (Path⁻) and formed colonies of abnormal morphology (Col⁻). This phenotype is similar to that of spontaneous variants of *P. tolaasii* strain S8501. The volatile toxin, tovsin, appeared to be produced at the level of the wild-type. Global regulation of secondary metabolite production in fluorescent pseudomonads has been reported. For example, inactivation of *lemA* in *Pseudomonas syringae* pv. *syringae* van Hall, a bacterial brown spot pathogen of bean, renders the bacterium defective in the production of pathogenic lipodepsipeptide toxins, i.e., syringomycins, as well as extracellular proteases (Hrabak and Willis, 1992). The phenotype of mutants of *P. tolaasii* strain PT814 is reminiscent of the LemA⁻ phenotype of *P. syringae* pv. *syringae*.

Like *lemA*, the cloned *rtpA* of *P. tolaasii* strain PT814 has conserved amino acid sequences of bacterial two-component regulators. In fact, it is predicted that the amino acid sequence of *rtpA* is more than 87% identical with that of PheN of *P. tolaasii* strain NCPPB1116, a brown blotch bacterium of *A. bisporus*, and ApdA of *P. fluorescens* strain Pf5, a biocontrol agent of fungal phytopathogens. In *P. tolaasii* strain NCPPB1116, PheN is required for the production of tolaasin and extracellular protease (Grewal et al., 1995), as observed in RtpA of *P. tolaasii* strain PT814. In *P. fluorescens* strain Pf5, ApdA controls the production of a variety of antifungal agents such as pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol, and tryptophan side chain oxidase (Corbell and Loper, 1995). As reported for the regulators of the *bvgS/lemA* family, the product of *rtpA* is predicted to have both sensor kinase and receiver domains. The lack of helix-turn-helix DNA binding motif at the receiver domain suggests that *rtpA* is not the sole regulator required for pathogenicity but a part of a complex regulatory system. It may be worthwhile comparing this system with the BvgS-A regulatory system of the whooping cough bacterium, *Bordetella pertussis* (Bergey et al.) Moreno-Lopez, in which biochemical control of a number of genes encoding pathogenic factors have been exten-

```
                B52▼
CTGGTCGCGGGCCATTTGCAGGCCCTGCTGGCCGTTCTGGCACCAGGTGACCTGAAAGCC  60
GCAACGTCCCAGATGCTCATGGACATAGGCGCCCAGCACGGGGTCATCTTCGATGGTCAG  120
GATACTGGGCAAGCCAACTGCTGCGGGATTCATTAGCGTCTGCAAGTCATTCTCAATCGC  180
TGATTATTCAAGATTAAGCCGTGCCAGGCAATCGCCAGCCGGCGGCTAATGGCCCGAAGA  240
       B1▼
TTGCCCCGTGTCATTAATTTGCAAGATTCCGCACCGCGCAAATGGCTACACTGCGCAGGT  300
                                            ******
GGCACGGGCCGGATGCCCGCGCGGGTCATTGAAAACAATGTGGTAGCAGGAGAGTGGCGT▶ 360
                                                            M
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GCTTAGAAGAATGGGGATAAAAGGCCGCGTACTGTTGCTGACCTTATTACCGACCAGCCT  420
 L  R  R  M  G  I  K  G  R  V L L L L T L L P T S L

GATGGCCTTGTTGTTGGGGGGCTATTTCACCTGGATGCAGCTCTCGGAGTTGCAGACCCA  480
 M A L L L L G G Y F T W M  Q  L  S  E  L  Q  T  Q
    PstI  B44▼
GTTGCTGCAGCGCGGCGAAATGATTGCCGAGCAGTTGGCGCCGTTGGTGGCTCCTGCCCT  540
 L  L  Q  R  G  E  M  I  A  E  Q  L  A  P  L  V  A  P  A  L

GAGCACGCGTAACACCGACCTGCTCGAACGCATCGCCACCCAGTCCCTTGAGCAACCGGA  600
 S  T  R  N  T  D  L  L  E  R  I  A  T  Q  S  L  E  Q  P  D

CGTGCGCGCCGTTTCGTTCCTCGCACCTGACCGTTCGCCCCTGGCCCACGCGGGCCCGAC  660
 V  R  A  V  S  F  L  A  P  D  R  S  P  L  A  H  A  G  P  T

CATGCTCAACCAGCCGCCGGTGGGCAACAGTTCGCATTTGTTGCAGCGTAGCGGCAATGA  720
 M  L  N  Q  P  P  V  G  N  S  S  H  L  L  Q  R  S  G  N  D

CGCCACCCGCTATCTGCTGCCGGTCTTTGGCCGCCACCGTAATCTTGCCGGCGAGCTGAT  780
 A  T  R  Y  L  L  P  V  F  G  R  H  R  N  L  A  G  E  L  I

TCCCGATGAGGCCGACCGCCTGCTGGGCTGGGTCGAGGTGGAGCTTTCCCACAACGGCAT  840
 P  D  E  A  D  R  L  L  G  W  V  E  V  E  L  S  H  N  G  M

GTTGCTGCGCGGCTACCGCAGCCTGTTCGCCAGCCTGCTGCTGATCGCCATCGGCCTGAT  900
 L  L  R  G  Y  R  S L F A S L L L I A I G L I

CTGCACCGCAGCCCTGGCCGTTGCGCATCAGTCGCACCATCAACTCGCCGATTGGCCAGAT  960
 C T A A L A L R I S R T I N S P I G Q I

CAAGCAGGCCGTGGCCCAGCTCAAGGACGGCAACCTCGAAACGCGCCTGCCGCCCCTCGG  1020
 K  Q  A  V  A  Q  L  K  D  G  N  L  E  T  R  L  P  P  L  G

CAGCCAGGAACTGGATCAATTGGCCTCGGGCATCAACCGCATGGCCGAAACCCTGCAAA  1080
 S  Q  E  L  D  Q  L  A  S  G  I  N  R  M  A  E  T  L  Q  N

TGCCCAGGAAGAATTGCAACACAGCATCGACCAGGCCACCGAAGACGTGCGCCAGAACCT  1140
 A  Q  E  E  L  Q  H  S  I  D  Q  A  T  E  D  V  R  Q  N  L

GGAAACCATTGAAATCCAGAACATCGAGCTGGACCTGACCCGCAAGGAGGCCCTGGAAGC  1200
 E  T  I  E  I  Q  N  I  E  L  D  L  T  R  K  E  A  L  E  A

CAGCCGCATCAAATCGGAGTTCCTGGCCAACATGAGCCACGAAATCCGCACTCCGCTCAA  1260
 S  R  I  K  S  E F L A N M S H E I R T P I N
                  PstI      H box
TGGCATCCTCGGCTTTACCCACCTGCTGCAGAAAAGCGAGCTGTCGCCGCGTCAGCTGGA  1320
 G I L G F T H L L Q K S E L S P R Q L D

TTACCTGGGCACCATCGAAAAGTCCGCCGACAACCTGCTGGGCATCATCAACGAGATTCT  1380
 Y  L  G  T  I  E  K  S  A  D  N  L  L  G  I  I  N  E  I  L

CGACTTCTCGAAAATCGAAGCCGGCAAGCTGGTGCTCGACAGCGTGCCGTTCAACCTGCG  1440
 D  F  S  K  I  E  A  G  K  L  V  L  D  S  V  P  F  N  L  R
   PstI
CGATTTGCTGCAGGACACCCTGACCATCCTCGCCCCCGCCGCCCATGCCAAGCAGCTCGA  1500
 D  L  L  Q  D  T  L  T  I  L  A  P  A  A  H  A  K  Q  L  E

ACTGGTCAGCCTGGTGTACCGCGACACGCCGCTGGCGCTGGTGGGCGACCCGTTGCGCCT  1560
 L  V  S  L  V  Y  R  D  T  P  L  A L V G D P L R L

CAAGCAGATCCTGACCAACCTGATCAGCAACGCGATCAAGTTCACCCGCGAAGGCACCAT  1620
 K Q I L T N L I S N A I K F T R E G T I
              N box
CGTCGCCCGCGCAATGGTTGAGGATGAAGAAGAAGACAGCGTACAACTGCGCATCAGTGT  1680
 V  A  R  A  M  V  E  D  E  E  E  D  S  V  Q L R I S V
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GCAAGACACCGGCATAGGCCTTTCCAATCAGGACGTGCGCGCGCTGTTCCAGGCGTTCAG  1740
 Q D T G I G L S N Q D V R A L F Q A F S
 D box                              F box
CCAGGCCGACAACTCGCTGTCGCGCCAACCCGGCGGCACCGGTTTGGGGCTGGTGATTTC  1800
 Q A D N S L S R Q P G G T G L G L V I S
                          G box  SalI
CAAACGCCTGATCGAGCAGATGGGCGGCGAGATTGGCGTCGACAGCACGCCAGGCGAAGG  1860
 K R L I E Q M G G E I G V D S T P G E G

CTCGGAGTTCTGGATCAGCCTGAACCTGCCCAAAACCCGCGATGACGTTGACGATTTGCC  1920
 S  E  F  W  I  S  L  N  L  P  K  T  R  D  D  V  D  D  L  P

TTCTGCGCCGCTGCTGGGCCGTCGTGTGGCGGTGCTGGAAAACCACGAGCTGGCGCGTCA  1980
 S  A  P  L  L  G  R  R  V  A  V  L  E  N  H  E  L  A  R  Q

GGGCCTTGCAACATCAGTTGGAAGACTGCGGCCTGGAAGTCACGCCGTTCAACACCCTCGA  2040
 A  L  Q  H  Q  L  E  D  C  G  L  E  V  T  P  F  N  T  L  E

AAGCCTGACCAACGGCATCACCAGCACCCATCAGACCGAACAGGCGATTGACCTGGCGGT  2100
 S  L  T  N  G  I  T  S  T  H  Q  T  E  Q  A  I  D  L  A  V
                                                     ▼E5
ACTCGGCCGTCACGGCCAACGACATCCCGCCCGAACGCCTCAACCAACACTTGTGGGACCT  2160
 L  G  V  T  A  N  D  I  P  P  E  R  L  N  Q  H  L  W  D  L

CGAACACCTGGGCTGCAAGGTCCTGGTGTTATGCCCGACCACCGAGCAAATGCTGTTCAA  2220
 E  H  L  G  C  K  V  L  V  L  C  P  T  T  E  Q  M  L  F  N

CCAGTCGGTGCCGAACCCCAACAGCCAATTGCAGGCCAAACCCGCGTGTACGCGTAAATT  2280
 Q  S  V  P  N  P  N  S  Q  L  Q  A  K  P  A  C  T  R  K  L

GCGTCGCGCCCTGGCTGACCTGATCAGCCCGCGCCCGTTGCGCAGCGAACCCGGCGAGCC  2340
 R  R  A  L  A  D  L  I  S  P  R  P  L  R  S  E  P  G  E  P

ACTGTCCAGCCGGGCACCGCGCGTGCTGTGCGTGGACGACAACCCGGCCAACCTGCTACT  2400
 L S S R A P R V L C V Q Q N P A N L L L
    ▼E1                  response regulator domain
GGTGCAAACCCTGCTCGAAGACATGGGCGCCAAGGTGCTCGCCGTCGAAAGTGGCTATGC  2460
 V Q T L L E D M G A K V L A V E S G Y A

GGCCATTGATGCGGTGAAGCAGGAAACCTTCGATCTGGTGCTGATGGACGTGCAAATGCC  2520
 A I D A V K Q E T F D L V L M Q V Q M Q

CGGCATGGACGGCCGCCAAAGTACCGAGGCGATTCGCCAGTGGGAGAGCGAGCGCCACGG  2580
 G M D Q R Q S T E A I R Q W E S E R H G

CACGCCACTGCCGGTGGTCGCCCTCACCGCCCACGCCATGGCCAACGAAAAACGCGCGCT  2640
 T P L P V V A L Q A H A M A N E K R A L

GCTGCAAAGCGGCATGGACGACTACCTGACCAAGCCCATCAGCGAACGGCAACTGGCCCA  2700
 L Q S Q M D D Y L T Q P I S E R Q L A Q

GGTGGTGCTGAAAATGGACCGGCCTGGCCCTGCGTAACCAGGGCCCCGAGCGCGGCAATGA  2760
 V V L K W T G L A L R N Q G P E R G N D

CAACCTCGGCCACGGCGTGCAACTGCTGGTGCTGGACCACGAGGAAGGCCTGCGCCTGGC  2820
 N  L  G  H  G  V  Q  L  L  V  L  D  H  E  E  G  L  R  L  A

GGCGAACAAGGCGGATCTCGCCGCCGATATGCTCGCCATGCTGCTGGCCTCCCTTGAAGC  2880
 A  N  K  A  D  L  A  A  D  M  L  A  M  L  L  A  S  L  E  A

CGACCGCTTGGCGATTACTGTCGCCCGCGAAGCCAAGGACAACAATGCGCTGATCGAACG  2940
 D  R  L  A  I  T  V  A  R  E  A  K  D  N  N  A L I E R
                                    conserved histidine region
CGTCCACCGCTTGCACGGCGCTACCCGCTACTGCGGCGTGCCGCAATTGCGCGCCGCCTG  3000
 V H R L H G A T R Y C G V P Q L R A A C

CCAGCGTGCGGAAACCTTGCTCAAGCAGGATGACGCCAAGGCCATGGCCGCGTTGGATGA  3060
 Q  R  A  E  T  L  L  K  Q  D  D  A  K  A  M  A  A  L  D  E

GCTGGACATGGCGATTGCGCGGCTGGCCAGTGAGGCTCGCGTCAACGCGTAGCTTTTCTG  3120
 L  D  M  A  I  A  R  L  A  S  E  A  R  V  N  A ■

TGGGAGCTGGTTTTTCTGTAGGGAACTGCTTTCCTGTGGGAGCTGGCTTGCCTGCGATGC  3180
AGGCGACTCGGTATGCCTGCCAGACCGAGGAGAGGCCATCGCAGGCAAGCCAGCTCCCAC  3240
ACAAACCAGCTTCCACAGGGGTACACGATCACCTTTAACCGTGCAGGGAGCCTTTCAATG  3300
```
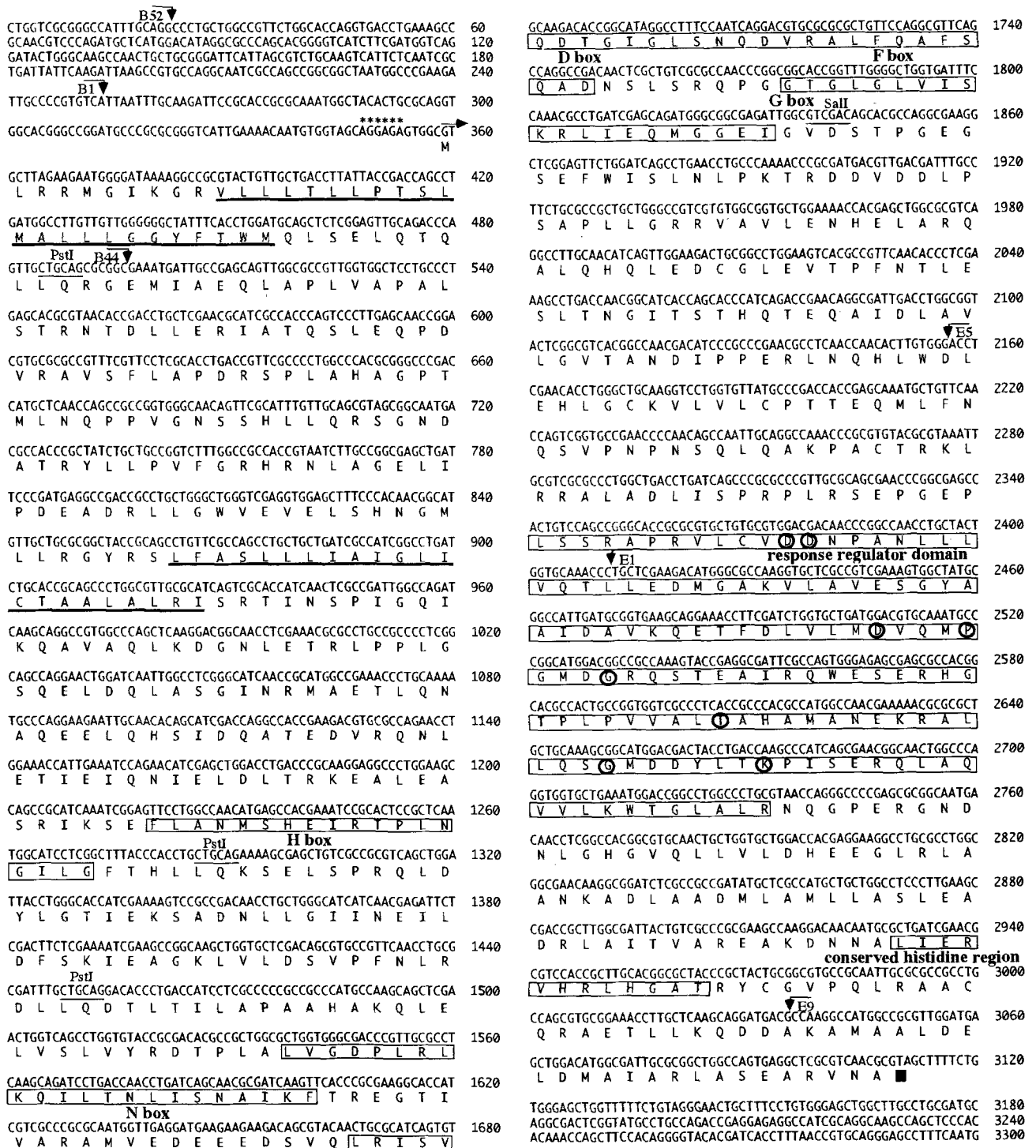
Fig. 7. Nucleotide sequence of the *rtpA* gene and flanking DNA.
The deduced amino acid sequence of *rtpA* is also given. The limits of deletion constructs corresponding to those shown in Fig. 4 are indicated by vertical arrows. The putative Shine-Dalgarno sequence (Shine and Dalgarno, 1974) and translation start site are marked by asterisks and a horizontal arrow, respectively. The unique (ATTAATTT) sequence present in the *rtpA* DNA with high GC content is indicated by a broken underline. Hydrophobic domains are underlined by a solid bar. The conserved H, N, D, F and G boxes of the predicted sensor kinase region, the response regulator domain and the conserved C-terminus of regulators belonging to the BvgS/LemA family are shown in boxes indicated as such (Stock et al., 1995; Uhl and Miller, 1995; Volz, 1995). Consensus amino acid residues in the response regulator domain are circled. The stop codon is indicated by the closed box at the end of the amino acid sequence.

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RtpA  1:MLRRMGIKGHVLLLTLLPTSLMALLLGGYFTWMQLSELQTQLLQRGEMIAEQLAPLVAPA 60
PheN  1:*********************T***************************************** 60
ApdA  1:**KKL****************V**********D***************************S***** 60

RtpA 61:LSTRNTDLLERIATQSLEQPDVRAVSFLAPDRSPLAHAGPTMLNQPPVGNSSHLLQRSGN 120
PheN 61:*********************************************R*********VAA*** 120
ApdA 61:MGNH**QM*******A***Q*****L*******L*****S***P**A*****MM****S 120

RtpA 121:DATRYLLPVFGRHRNLAGEIPDEADRLLGWVEVELSHNGMLLRGYRSLFASLLLIAIGL 180
PheN 121:*************************A**************************************** 180
ApdA 121:*****Q***********D*****S*********LD***S*******N*********PA** 180

RtpA 181:ICTAALALRISRTINSPIGQIKQAVAQLKDGNLETRLPPLGSQELDQLASGINRMAETLQ 240
PheN 181:*******V***************************p**********C*A*************** 240
ApdA 181:AV**L**V*WG****N*LT*********************************E*********S*** 240

RtpA 241:NAQEELQHSIDQATEDVRQNLETIEIQNIELDLTRKEALEASRIKSHFLANMSHEIRTPL 300
PheN 241:******************************************A*************************** 300
ApdA 241:***********************************A********************************** 300
MCR in H box                                       FLANMSHEIRTPL

RtpA 301:NGILQFTHLLQKSELSPRQLDYLGTIEKSADNLLGIINEILDFSKIEAGKLVLDSVPFNL 360
PheN 301:**********************HV************************************** 360
ApdA 301:*****************T****************S*********************I***** 360
         NGILG

RtpA 361:RDLLQDTLTILAPAAHAKQLELVSLVYRDTPLALVGDPLRLKQILTNLISNAIKFIREGT 420
PheN 361:***********************************V************************* 420
ApdA 361:***************************Q*****************V*****I***** 420
MCR in N box                       LLGDPLRLKQILSNLLSNAIKF

RtpA 421:IVARAMVEDEEEDSVQLRISVQDTGIGLSNQDVRALFQAFSQADNSLSRQPGGTGLGLVI 480
PheN 421:**********************************TE*********************** 480
ApdA 421:******L*E*H*****I***********************I********** 480
MCR in D/F and G boxes   LRIEVTDTGPGLSEEDIEQLFEAFYQAD        GTGLGLAI

RtpA 481:SKRLIEQMGGEIGVDSTPGEGSEFWISLNLPKTRDDVDDLPSAPLLGRRVAVLENHELAR 540
PheN 481:************************************************DV** 540
ApdA 481:*****************************************AE***GP*************** 540
         SKRLIKQMGGRI

RtpA 541:QALQHQLEDCGLEVTPFNTLESLTNGITSTHQTEQAIDLAVLGVTANDIPPERLNQHLWD 600
PheN 541:*********************************************************** 600
ApdA 541:*****************A******GV**S***********I*T**MS****S**I** 600

RtpA 601:LEHLGCKVLVLCPTTEQMLFNQSVPNPNSQLQAKPACTRKLRRALADLISPRPLRSEPGE 660
PheN 601:******************************TP***********-*VAQRTR* 659
ApdA 601:***********************T**HL*****H****************S**VT**RA****E* 660
MCR in response regulator domain

RtpA 661:PLSSRAPRVLCVDDNPANLLLVQTLLEDMGAKVLAVESGYAAIDAVKQETFDLVLMDVQH 720
PheN 660:********************************************************* 719
ApdA 661:T********************I***********DN****LN*IQT*P****M***** 720
         P SGEAMRVLLLDDHE LLL Q LLEDLGFAVDAVASGAAAVDAVKTE F LVLMDVMM

RtpA 721:PGMDGRQSTEAIRQWESERHGTPLPVVALTAHAMANEKRALLQSGMDDYLTKPISERQLA 780
PheN 720:***R***************************************************** 779
ApdA 721:****G*K*****************I********************************* 780
         PGMDGI   EAIRQIKQ R G PLPVVFLTAHAELPDKIAALQLGADDYLTKPFSLRRLV

RtpA 781:QVVLKWTGLALRNQGPERGNDNLGHGVQLLVLDHEEGLRLAANKADLAADMLAMLLASLE 840
PheN 780:****************************A***************** 838
ApdA 781:*************W******ASERPEL*LE*Q***QD******G***************D 840
         QAV KALAEGARR

RtpA 841:ADRLAITVAREAKDNNALIERVHRLHGATRYCGVPQLRAACQRAETLLKQDDAKAMAALD 900
PheN 839:************************I*******AE***************QGHG*** 898
ApdA 841:***E***KA**A*N*Q**************************S******E****F***** 900
MCR in histidine region   @----H+@+G

RtpA 901:ELDMAIARLASEARVNA                                        917
PheN 899:******G-A*GQ-----                                        909
ApdA 901:***H**G***A***T**                                        917
```

Fig. 8. Amino acid sequence alignments among RtpA of *Pseudomonas tolaasii* strain PT814, PheN of *P. tolaasii* strain NCPPB1116 (Grewal et al., 1995), and ApdA of *Pseudomonas fluorescens* Pf5 (Corbell and Loper, 1995).

Analysis was conducted by using GENETYX ver. 8 computer software (Software Development, Tokyo). Amino acid sequences predicted to have characteristics of trans-membrane regions (positions 11 to 33 and 169 to 180), the sensor kinase (H box, positions 288 to 305; N box, 394 to 415; D/F box, 437 to 464; G box, 473 to 492), the response regulator (positions 661 to 792), and the conserved histidine region (positions 858 to 869) are shown in boxes. Examples of *most* conserved *r*esidues reported (MCR: Stock et al., 1995; Uhl and Miller, 1995; Volz, 1995) are also given. Symbols: *, amino acid residues identical with those of RtpA; —, gaps in the alignments; @, hydrophobic amino acid residues; +, positively charged amino acids.

sively investigated to identify the *bvgS/lemA* family (Uhl and Miller, 1995). In the model, the 134 kDa BvgS protein recognizes signals in the periplasmic domain located between the two transmembrane anchors. Signals are subsequently transmitted by a phosphorylation cascade through the cytoplasmic domain containing the histidine protein kinase, the receiver domain, and the C-terminal histidine regions, to the 24 kDa BvgA protein, which is a typical response regulator containing both phosphorylation and DNA-binding sites. In *P. fluorescens* strain CHO1, *gacA* was also identified as a member of the *fixJ/degU* family of response regulators that control the production of a variety of antifungal antibiotics (Laville et al., 1992). No report, however, has yet described the presence of both *lemA* and *gacA* in the same strain of *P. fluorescens*, nor the presence of *gacA* in *P. tolaasii* strains. We are currently characterizing mutants generated by mini-Tn5km1 insertions that phenotypically belong to RtpA⁻ but are not complemented by the cloned *rtpA* gene. mini-Tn5km1 insertion sites in these mutants may be detected at other regulatory loci which could directly target tolaasin biosynthetic genes. Further analysis may reveal other components involved in the pathogenicity of the bacterium.

In terms of signals, our preliminary study showed that tolaasin production in *P. tolaasii* strain PT814 is activated by components of fruiting bodies of *P. ostreatus*, although the molecules have not yet been identified (Murata and Magae, 1996). A precedent of the recognition of host signals by plant pathogenic pseudomonads is observed in *P. syringae* pv. *syringae* strain B3AR132, which produces syringomycin, a toxin chemically and functionally related to tolaasin (Mo and Gross, 1991). The expression of *syrB* of *P. syringae* pv. *syringae* strain B3AR132, which is involved in the biosynthesis of syringomycin, has been reported to be induced to a great extent by a combination of some of the phenolic glucosides and sugars present in the host plants (Mo and Gross, 1991). Whether *P. tolaasii* responds to the signals peculiar to *P. ostreatus* but chemically similar to those found in the system of *P. syringae* pv. *syringae* should be determined by studying the fungus-bacterium interactions.

Based on the mutant phenotype and predicted amino acid sequence, *rtpA* of *P. tolaasii* strain PT814 may be the allele of *pheN* of NCPPB1116. It is interesting to note, however, that *rtpA* is expected to encode a 100.6 kDa protein consisting of 917 amino acid residues, whereas *pheN* encodes an 83 kDa protein consisting of 909 amino acid residues (Grewal et al., 1995). In addition, *P. tolaasii* strain NCPPB1116 has been reported to produce in PAF a sector of nonpathogenic variants with distinct morphology from that of the wild-type colony, and this phenotype is attributed to the spontaneous duplication of 661 bp DNA within the *pheN* gene (Han et al., 1997). In contrast, the pathogenicity of *P. tolaasii* strain PT814, in which the variant sector seldom occurs, is genetically stable. Whether the structure and functions of this type of regulators are shared by a wider range of isolates of each species should be further inves-

tigated.

It is interesting to note that *P. tolaasii* strain S8501 generates avirulent variants in PF-broth, and these variants tend to overwhelm the parent. This incidence is, however, significantly reduced in the presence of *P. ostreatus* extracts or at a temperature lower than 22°C. This observation indicates how a saprophytic bacterium could evolve from a pathogenic bacterium. "*Pseudomonas reactans*," which is saprophytic but not pathogenic to cultivated mushrooms, produces a lipodepsipeptide compound chemically related to tolaasin of *P. tolaasii* (Mortishire-Smith et al., 1991). Differences in the growth environments of *P. tolaasii* may allow the bacterial population to select symbiotic systems, saprophytic or parasitic, through mutation and selection mechanisms. As the rate of occurrence of variants depends upon the growth conditions, it is conceivable that environmental stimuli including metabolites of hosts as well as the pathogen itself may play a role in the induction of the variants. It is noteworthy that the isolates of *P. tolaasii* show a wide diversity in the rate of avirulent variant formation. *Pseudomonas tolaasii* strain PT814 is very stable in contrast to *P. tolaasii* strains S8501 and NCPPB1116 (this report; Grewal et al., 1995). *Pseudomonas tolaasii* has been shown to be of a sizable genetic diversity (Thorn and Tsuneda, 1996), and it displays variability even in the rate of spontaneous mutation. Although tolaasin is generally considered to be the primary determinant of the pathogenicity of *P. tolaasii* (Murata and Magae, 1996; Rainey et al., 1993; Shirata et al., 1995), the rate of occurrence of variants as well as the complex interactions with host factors in pathogenic processes specifying host-parasite relations may also play a major role in the parasitism. Shirata et al. (1995) noted, for example, that although tolaasins and tovsin can cause severe damage to many plant species, no instance has yet been reported of plant diseases caused by *P. tolaasii*. Further investigations of host factors and other regulatory elements involved in the pathogenicity and genetic variation of the bacterium may clarify important aspects of mushroom-pathogen interactions, eventually leading to the development of new methods of controlling the disease.

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