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Short Communication

Molecular cloning of *glpFKRD* of a mushroom-pathogenic bacterium, *Pseudomonas tolaasii* strain PT814: induction of an avirulent mutant carrying a single mini-Tn*5*km1 insertion in *glpD*.

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Pseudomonas tolaasii **strain PT814 causes brown blotch disease in cultivated mushrooms. A pleiotropic avirulent mutant was isolated by mini-Tn*5*km1 insertion mutagenesis. The insertion was localized in an open reading frame (ORF) predicted to encode *sn*-glycerol-3-phosphate dehydrogenase (*glpD*). ORFs that should encode its regulator, kinase, and facilitator were also identified as the *glp* gene cluster in the bacterium. The data suggest that the *glp* system may contribute to the ecology of this pathogen.**

Key Words——*glpD*::mini-Tn*5*km1; *glpFKRD*; pathogenicity; *Pleurotus ostreatus*; *Pseudomonas tolaasii*.

Pseudomonas tolaasii Paine, which is highly capable of colonizing cultivated mushrooms and the mushroom culture environment (Murata, 1998; Suyama and Fujii, 1993), often devastates the production of fruiting bodies of *Pleurotus ostreatus* (Jacq.: Fr.) Kummer and *Agaricus bisporus* (Lange) Singer (Suyama and Fujii, 1993; Tolaas, 1915). The bacterium produces extracellular lipodepsipeptide toxins, tolaasins, that induce cell death of the host and a volatile toxin, tovsin, that stimulates rotting of the mycelia (Murata and Magae, 1996; Nutkins et al., 1991; Shirata, 1996). Molecular analysis of host-parasite interactions has revealed that tolaasin production and other pathogenic traits in the bacterium are controlled by signals from hosts and regulators present in the bacterium that are predicted to respond to such stimuli (Grewal et al., 1995; Murata, 1999; Murata et al., 1998; Murata and Magae, 1996). Previously, the *lemA* family of two-component sensor kinases required for tolaasin production and *rpoS* encoding the stress response sigma factor were cloned and characterized (Grewal et al., 1995; Murata, 1999; Murata et al., 1998). In this paper, a mutant of *P. tolaasii* strain PT814 carrying a single transposon (mini-Tn*5*km1) insertion in the *sn*-glycerol-3-phosphate dehydrogenase gene (*glpD*) present in a cluster of genes involved in glycerol metabolism (*glpFKRD*) was shown to be altered in pleiotropic phenotypes that determine its pathogenicity and ecology.

A transposon, mini-Tn*5*km1, encoding the kanamycin resistance gene (Km^r) was introduced into *P. tolaasii* strain PT814 [prototroph, naturally resistant to ampicillin (Ap), chloramphenicol (Cm), gentamicin, spectinomycin and streptomycin] by mating with *Escherichia coli* (Migula) Castellani and Chalmers strain S17-1λ*pir* carrying the pUT::mini-Tn*5*km1 as described previously (De Lorenzo et al., 1990; Murata et al., 1998). Transconjugants selected on *Pseudomonas* agar F (PAF; Difco, Detroit, MI) containing Km (100 μg/ml) and Cm (100 μg/ml) were inoculated into the fruiting bodies of *P. ostreatus* by using sterile toothpicks, and the extent of development of disease symptoms was visually determined (Fig. 1; Tsukamoto et al., 1998). Of 2500 transconjugants exhibiting Km^r Cm^r, 19 strains failed to induce brown blotch in *P. ostreatus* fruiting bodies (Path$^-$; Murata et al., 1998). Of these, *P. tolaasii* strain MUR39 behaves differently from others by exhibiting an extremely slow growth and rough colony morphology on PAF (Fig. 1). A reduced growth rate was also noted in a medium containing glucose as a carbon source (Table 1). The mutant is pleiotropically defective in the production of tolaasin (Tox$^-$) in minimal salts succinate medium containing water extracts of *P. ostreatus* fruiting bodies (Murata and Magae, 1996) and extracellular protease (Prt$^-$) in PF-broth (=PAF without agar) and in utilization of glycerol as a carbon source, and insensitive to tetracycline (Table 1). The production of tovsin and the response to stresses such as hydrogen peroxide and UV-radiation during interaction with the host, however, were not affected by the mutation (Table 1; Murata, 1998).

A single mini-Tn*5*km1 insertion was identified in *P. tolaasii* strain MUR39 by Southern hybridization using a probe of the 1.8 kb *Eco*RI fragment of pUT::mini-Tn*5*km1 (Murata et al., 1998) wherein an intense hybridization

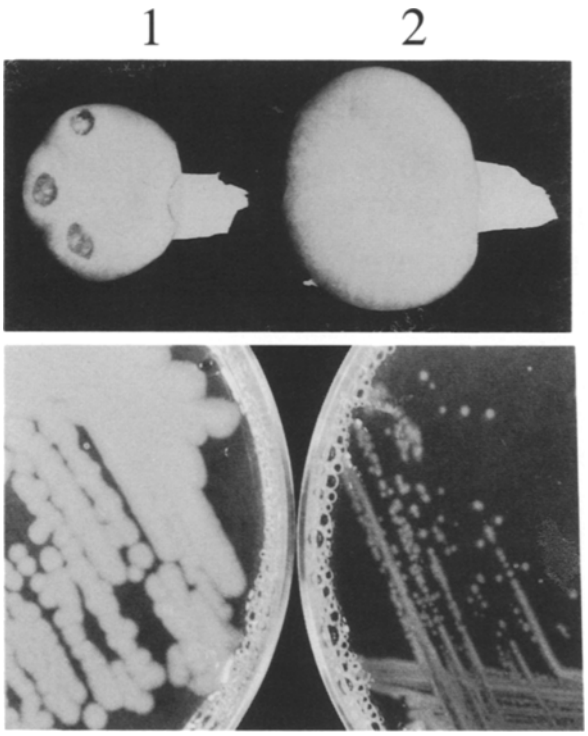


Fig. 1. Pathogenicity and colony morphology of *P. tolaasii* strain MUR39 carrying a chromosomal *glpD*::mini-Tn*5*km1 and the parent, *P. tolaasii* strain PT814. Assay conditions are detailed in the text. The upper panel and the lower panel depict the development of brown blotch on *P. ostreatus* fruiting bodies, and growth and colony morphology on PAF, respectively. Lane 1, *P. tolaasii* strain PT814; lane 2, *P. tolaasii* strain MUR39.

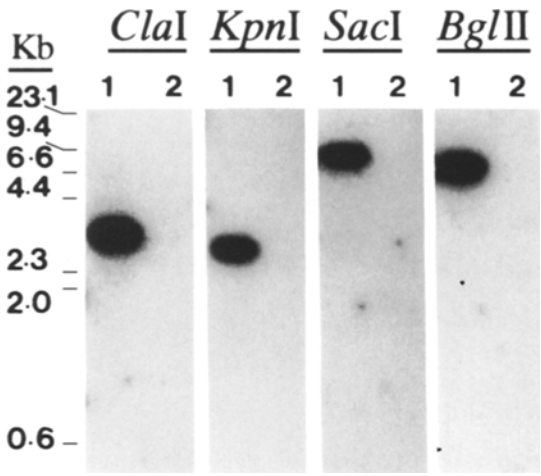


Fig. 2. Southern hybridization analysis of a mini-Tn*5*km1 insertion mutant, *Pseudomonas tolaasii* strain MUR39. The 1.8 kb *Eco*RI fragment of pUT::mini-Tn*5*km1 (De Lorenzo et al., 1990) labeled with [^{32}P]dCTP was used as a probe. Lane 1, *P. tolaasii* strain MUR39; Lane 2, *P. tolaasii* strain PT814. Molecular size is also given.

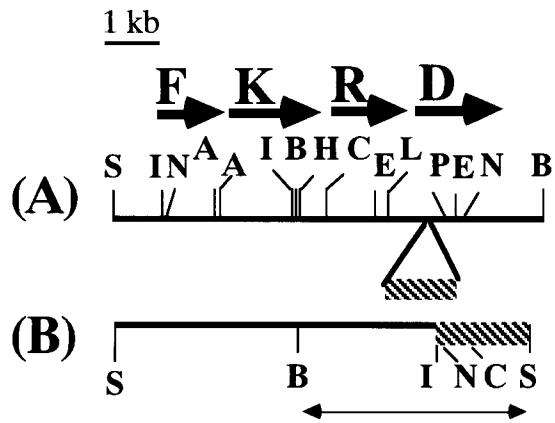


Fig. 3. Restriction endonuclease map of the 8.2 kb *Sac*I-*Bgl*II DNA containing *glpFKRD*, and schematic representation of the mini-Tn*5*km1 insertion identified in the mutant genome. Thick parallel lines depict the 8.2 kb *Sac*I-*Bgl*II DNA present in pHHM105 (A) and the 6.1 kb *Sac*I DNA in pHHM101 (B), respectively. The hatched bar (B) or open triangle with the hatched bar (A) indicates the mini-Tn*5*km1 insertion identified in the genome of *P. tolaasii* strain MUR39. The location and the predicted direction of *glpFKRD* transcription are indicated by the arrows marked F, K, R and D for *glp*F, *glp*K, *glp*R and *glp*D, respectively (A). The arrow in (B) indicates the fragment used for a probe to screen the wild-type allele of mutated genetic locus. Abbreviations: A, *Apa*I; B, *Bgl*II; C, *Cla*I; E, *Eco*RV; H, *Bam*HI; I, *Eco*RI; L, *Sal*I; N, *Kpn*I; P, *Pst*I; S, *Sac*I.

signal due to the presence of two signals was detected in samples of the mutant digested with *Cla*I, of which the restriction site is within the transposon (Figs. 2, 3). A single hybridization signal was noted in genomic digests with *Sac*I and *Kpn*I in association with the sites located near one end of the transposon, and with *Bgl*II, whose site is not present within the transposon (Figs. 2, 3). The size of hybridized fragments matched well with ones expected from the cloned fragment carrying mini-Tn*5*km1 and its wild-type allele (Figs. 2, 3). For example, hybridization signals in the sample digested with *Bgl*II, *Cla*I, *Kpn*I, and *Sac*I appeared as a band of 6.6 kb, 2.8 kb, 2.4 kb, and 7.8 kb, respectively, which corresponded to the sum of ca. 4.8 kb of the wild-type allele plus ca. 1.8 kb of the mini-Tn*5*km1 DNA, ca. 2.2 kb of the allele plus ca. 0.6 kb of the mini-Tn*5*km1 DNA, ca. 0.6 kb of the allele plus ca. 1.8 kb of the mini-Tn*5*km1 DNA, and ca. 6.0 kb of the allele plus ca. 1.8 kb of the mini-Tn*5*km1 DNA, respectively (Figs. 2, 3). No hybridization signal was observed in samples of *P. tolaasii* strain PT814. In addition, by using the 6.5 kb *Kpn*I-*Sac*I *rtpA* DNA as a probe, the insertion was found to occur at a genetic locus other than *rtpA*, a regulatory gene previously reported to control a pleiotropic phenotype in *P. tolaasii* strain PT814 (Murata et al., 1998).

To identify the mutated gene, an attempt was made to clone a genetic locus disrupted by mini-Tn*5*km1 in *P. tolaasii* strain MUR39 by using the Km^r marker. This approach was taken, rather than screening a genomic library of *P. tolaasii* strain PT814 for complementation, because no vector was available to conduct transconju-

Table 1. Characteristics of *P. tolaasii* strain MUR39 carrying *glpD*::mini-Tn*5*km1 and its parent, *P. tolaasii* strain PT814.

Characteristics	*P. tolaasii* strain:	
	PT814	MUR39
Pathogenicity[a]	+	−
Production of[b]:		
Tolaasin	16	0
Protease	32	0
Tovsin	+	+
Utilization of glycerol as a carbon source[c]	+	−
Doubling time (min) in minimal salts glucose medium[d]	120	280
Sensitivity to Tc (μg/ml)[e]	50	>1000
Resistance to[f]:		
UV (600 erg)	0.83	0.74
H_2O_2 (1.2 M/30 min)	0.97	0.95

a) Development of brown blotch on *P. ostreatus* fruiting bodies (Fig. 1) was visually determined as described previously (Murata et al., 1998; Tsukamoto et al., 1998). +: brown blotch, −: no symptom.

b) Levels of tolaasin and protease were semiquantitiatively assayed by using potato tuber slices and 3% gelatyin plate with serially diluted samples, and expressed as relative units (Murata et al., 1998; Tsukamoto et al., 1998). Tovisin production was determined by observing the inhibition of germination of *Kactuca sativa* L. seedlings (Murata et al., 1998). +: tovsin production.

c) +: growth (OD_{600} value of 2.5) in minimal salts-glycerol medium on incubation at 24°C for 24 h. −: no growth (OD_{600} value of 0.07) under the assay conditions.

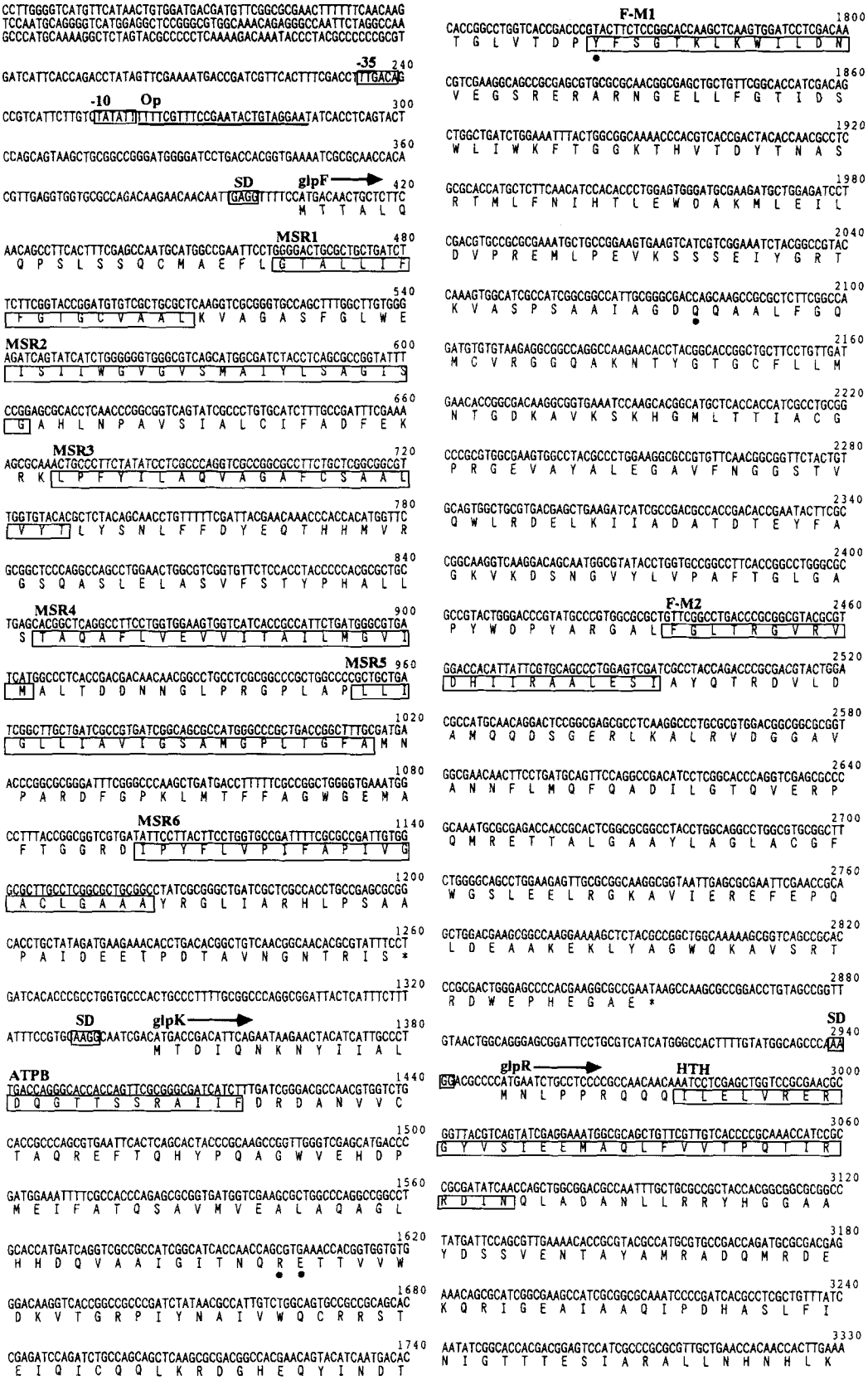
d) Doubling time (min) of bacteria during the exponential growth phase in minimal salts-glucose medium at 24°C.

e) The least conc. of tetracyclin (Tc) that inhibits growth of bacteria.

f) Viable cell numbers after exposure to stress are expressed relative to those of sample without stress. For details of assay conditions, see Murata (1998).

gation in this mutant due to the drug marker problem. The genomic DNA of the mutant was digested with restriction endonuclease *Sac*I, which should not cut the transposon (De Lorenzo et al., 1990), ligated into the *Sac*I site of pBluescript SK$^+$ (Stratagene, La Jolla, CA), and introduced into *E. coli* strain JM109. Transformants were selected on LB agar containing Km (50 μg/ml) and Ap (50 μg/ml), from which a plasmid was isolated and analyzed by restriction and Southern hybridization analyses to examine the extent of recombination. The analyses showed that only one side of the DNA flanking region of the transposon could be cloned and not both sides, due to the presence of the *Sac*I site at one end of the transposon not described in the literature but identified in later analysis (De Lorenzo et al., 1990; Murata et al., 1998). This plasmid was designated as pHHM101. Subsequently, pHHM105 carrying the wild-type allele was identified in a genomic library of *P. tolaasii* strain PT814 constructed in a cosmid, pLAFR5 (Tc^r), by colony hybridization using the 4.7 kb *Bgl*II-*Sac*I fragment of pHHM101 labeled with [α-P^{32}]dCTP as a probe (Fig. 3; Murata et al., 1998).

Nucleotide sequencing was performed on subclones and deletion derivatives of pHHM101 and pHHM105 by using ABI prism 377 sequencer (Perkin Elmer Japan, Urayasu), and the data were analyzed using the GENETYX Mac ver 8.5 computer software (Software Development Co. Ltd, Tokyo) and Advanced Blast Search provided by The National Center for Biotechnology (http://www.ncbi.nlm.nih.gov/). The sequence of mini-Tn*5*km1 was identified in a genetic locus cloned in pHHM101 which corresponded to an open reading frame (ORF) of 1542 bp present in pHHM105 (Fig. 4). The ORF is sufficient to encode 514 amino acid residues with a molecular mass of 57.0 kDa and pI. of 9.1 (Fig. 3 and 4; GenBank/EMBL/ DDBJ accession number AB015976). The putative gene product encoded in the ORF has an amino acid sequence 68% identical with (79% similar to) that of *sn*-glycerol-3-phosphate dehydrogenase (GlpD) reported in *Pseudomonas aeruginosa* (Schroeter) Migula (Fig. 4; Schweizer and Po, 1994). Upstream of the putative *glpD* translational start site, ORFs of 753 bp, 1512 bp, and 855 bp were identified, which are sufficient to encode a protein of 251 amino acid residues with a molecular mass of 27.7 kDa with pI. of 5.28, 55.7 kDa with pI. of 5.43, and 30 kDa with pI. of 5.37, respectively (Figs. 3, 4; GenBank/EMBL/DDBJ accession number AB015975, AB015974 and AB015973, respectively). The amino acid sequence of putative 27.7 kDa, 55.7 kDa, and 30 kDa products exhibits 74% identity


-35
-10
Op
SD
glpF
MSR1
MSR2
MSR3
MSR4
MSR5
MSR6
F-M1
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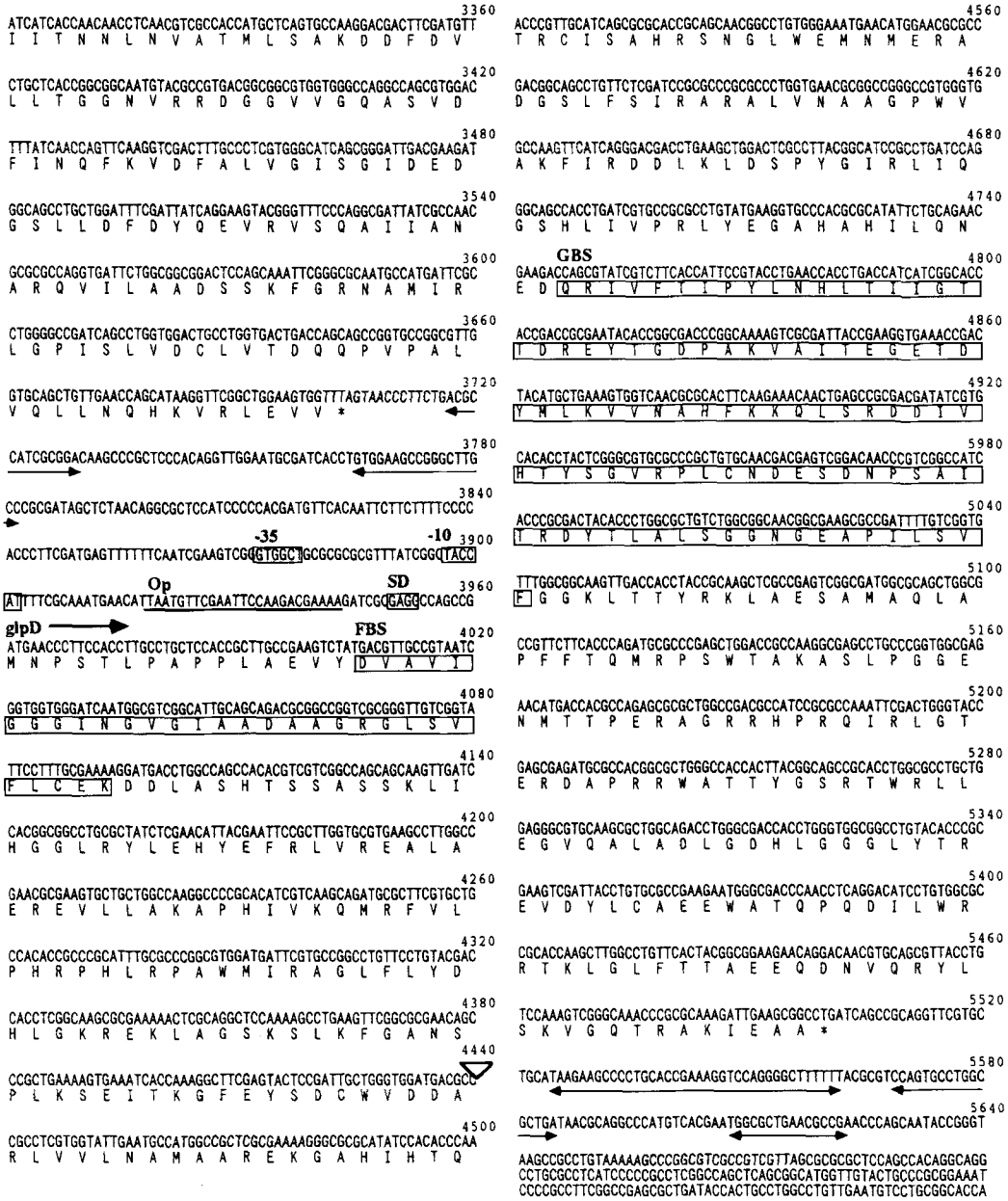

Fig. 4. Nucleotide sequence of *glpFKRD* and flanking DNA region. The deduced amino acid sequence with the translation stop site (asterisks) of each ORF is also given. The mini-Tn*5*km1 insertion site corresponding to that shown in Fig. 3 is indicated by the open triangle at 4440 bp. The putative Shine-Dalgarno sequence (SD; Shine and Dalgarno, 1974), and -35 and -10 promoter regions are boxed in the nucleotide sequence. Putative operators commonly identified in *glp* genes of other bacteria (Op; Schweizer and Po, 1996; Schweizer et al., 1997) and palindromes are marked with solid lines and bi-directional arrows, respectively, below the nucleotide sequence. Location and direction of each ORF are indicated by unidirectional arrows with descriptions above the nucleotide sequence. Predicted features of the amino acid sequence are boxed in with descriptions in abbreviations, e.g., membrane-spanning regions (MRS) in GlpF, ATP-binding motif (ATPB), and motifs 1 and 2 of FGGY family of carbohydrate-binding proteins (F-M) in GlpK, helix-turn-helix DNA binding motif (HTH) in GlpR, and flavin-binding site (FBS) and glycerol-3-phosphate binding site (GBS) in GlpD. Amino acid residues conserved in glycerol binding in GlpK are indicated by dots.

with (85% similarity to) the regulator (GlpR), 77% identity with (85% similarity to) the kinase (GlpK) and 75% identity with (81% similarity to) the diffusion facilitator (GlpF), respectively, encoded in the *glp* cluster of *P. aeruginosa* (Fig. 4; Schweizer and Po, 1996; Schweizer et al., 1997).

Glycerol and its complex metabolism are reported to play an important role in pseudomonads, in which the *glp* system is not only responsible for salvaging the glycerol moiety of its own degraded phospholipids but is also involved in the expression of characteristics that determine their ecology (Schweizer et al., 1997). For instance, the

glpFKRD system in an opportunistic human pathogen, *P. aeruginosa*, traps glycerol in the extracellular milieu into the cytoplasm as glycerol-3-phosphate and converts it into DHAP to be utilized for energy metabolism, biosynthesis of phospholipids, and the production of pathogenic factor, alginate. In the parasitic state of *P. aeruginosa*, glycerol is supplied in the form of phospholipids from the host such as phosphatidylcholine, a major component of human lung surfactant (Schweizer et al., 1997).

The similarity in the organization of the gene cluster and the putative gene products suggests that *P. tolaasii* strain PT814 reported here should have a glycerol metabolic pathway very similar to that of *P. aeruginosa*. It is interesting to note, however, that in terms of host-parasite interactions, *P. ostreatus* contains a significant amount of free glycerol (0.4% in dry weight; Yoshida et al., 1984). Previously, I and my coworkers reported that sugars present in *P. ostreatus* such as trehalose, which is abundant in the host (8.1% in dry weight; Yoshida et al., 1984), stimulate tolaasin production in the bacterium (Murata and Magae, 1996; Murata et al., 1998). While sugars present in the host could play an important role as a signal in the production of pathogenic factors and other secondary metabolites, it is also worth examining the contribution of sugars as well as the mechanisms involved in the sugar metabolism as components required for their house-keeping process.

Presently it is unclear precisely how the *glp* system controls these features. However, it is plausible to speculate that glycerol is one of the major energy sources that confers a balanced metabolic status, particularly in the process of host-parasite interactions, and/or that it is involved in the biosynthesis of cell components such as those containing a glycerol moiety. An example of such components, which are biologically most critical, may be the phospholipids of the bacterial cell membrane, where transcription/translation of genes occurs, toxins, proteins and other extracellular products are excreted, and exotic compounds such as antibiotics are secreted. The data presented here and reported previously underscore the importance of the sugars present in *P. ostreatus* during the host-parasite interaction in a mushroom-pathogenic bacterium, *P. tolaasii*. The phenomena observed offer a clue to approaches for analyzing molecular mechanisms of the pathogenic process.

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