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Molecular genetic characterization of bacterial isolates causing brown blotch on cultivated mushrooms in Japan

Greg Thorn[1),*] and Akihiko Tsuneda[2)]

[1)]Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824, U.S.A.
[2)]Tottori Mycological Institute, 211 Kokoge, Tottori 689–11, Japan

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The polymerase chain-reaction (PCR) was used to amplify 16S ribosomal DNA (16S rDNA) from bacteria, identified as *Pseudomonas tolaasii* or *P. fluorescens*, causing brown blotch on cultivated mushrooms in Japan. PCR-amplified 16S rDNA was analyzed on the basis of nucleotide sequence and restriction fragment length polymorphisms (RFLP) to determine the specific identity of isolates. Banding patterns obtained through PCR using primers corresponding to repetitive extragenic palindromic sequences of enteric bacteria (REP-PCR) were used to determine the relatedness of conspecific isolates. All *P. tolaasii* isolates and a mushroom pathogen identified as *P. fluorescens* had identical RFLP patterns and partial 16S sequences, and are considered conspecific. An isolate of *P. fluorescens* from creamery wastes (IFO 3507) differed slightly from isolates of *P. tolaasii* in both 16S sequence (0.8%) and RFLP patterns (d=0.08), and had almost entirely different REP-PCR bands (d=0.88–1.0). Phylogenetic analyses based on 16S sequences indicated that *P. tolaasii* and *P. fluorescens* are close members of *Pseudomonas* sensu stricto. REP-PCR shows promise in characterizing isolates pathogenic on different mushroom crops. Two isolates of *P. tolaasii* pathogenic on *Pleurotus ostreatus* had identical banding patterns, but three isolates from *Lentinula edodes* showed the greatest diversity.

Key Words—*Agaricus brunnescens*; bacterial disease; *Lentinula edodes*; *Pseudomonas fluorescens*; *Pseudomonas tolaasii*; REP-PCR.

The bacterium *Pseudomonas tolaasii* Paine causes the brown blotch disease of commercial mushrooms (Tolaas, 1915; Paine, 1919). This disease causes significant losses worldwide in crops of *Agaricus brunnescens* Peck (=*A. bisporus* (Lange) Imbach, the button mushroom) (Fermor et al., 1991) and has been noted in the most important mushroom crops in Japan, namely, *Flammulina velutipes* (Curt.: Fr.) Singer (enokitake), *Lentinula edodes* (Berk.) Pegler (shiitake), and *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (the oyster mushroom or hiratake) (Suyama and Fujii, 1993; Gill, 1995; Tsuneda et al., 1995). However, these commercially cultivated mushrooms and a number of other ligninolytic basidiomycetes are capable of attacking and degrading living bacteria in vitro (Barron and Thorn, 1987; Barron, 1988), including *P. tolaasii* (Thorn and Tsuneda, 1992, 1993). Differences have been noted in the outcomes of interactions between various strains of *L. edodes* and strains of *P. tolaasii* isolated from different mushroom hosts. These differences included varying degrees of pathogenicity to *L. edodes*, and also susceptibility to inhibition, lysis and attack by *L. edodes* in vitro (Tsuneda and Thorn, 1994).

Although *P. tolaasii* is included in the Approved Lists of Bacterial Names (Skerman et al., 1989), its validity as a distinct species and its place in *Pseudomonas* have not been fully resolved. In Bergey's Manual of Systematic Bacteriology (Palleroni, 1984), *P. tolaasii* is included in *Pseudomonas* Section V, which "includes a number of species of *Pseudomonas* whose natural relationships with well characterized species of the genus are largely unknown." *Psendomonas tolaasii* has traditionally been characterized as a fluorescent pseudomonad causing brown blotch on mushrooms (Paine, 1919) and a white line reaction when grown with "*Pseudomonas reactans*" on agar media (Wong and Preece, 1979). Young et al. (1978) listed *P. tolaasii* among the fluorescent species of *Pseudomonas* that are distinguishable as species. DNA:23S rRNA hybridization experiments (de Vos et al., 1985) place *P. tolaasii* in Section I of *Pseudomonas* (Pelleroni, 1984), with *P. fluorescens* Migula. Similarities in the sequence of the gene encoding the outer-membrane protein OprF in both *P. fluorescens* and *P. tolaasii* also support the close relatedness of these two species (de Mot et al., 1994). Substrate utilization tests, electrophoresis of soluble proteins, and DNA:DNA hybridization experiments among *P. tolaasii* strains showed that the species can be differentiated from both *P. fluorescens* and "*P. reactans*" even though some strains may not give a white line reaction, nor be pathogenic on mushrooms (Goor et al., 1986).

Knowledge of the specific identity and relatedness of

Contribution No. 312 of the Tottori Mycological Institute, Tottori, Japan.

*Present address: Department of Botany, University of Wyoming, Laramie, WY 82071, U.S.A.

isolates of bacteria causing brown blotch on cultivated mushrooms in Japan could be valuable in planning for improved mushroom culture, particularly in assessing the methods of bacterial transmission, the virulence of bacterial strains and the susceptibility of particular mushroom crops. We studied the genetic relatedness of seven isolates identified as *P. tolaasii*, isolated from a variety of mushroom hosts in Japan, and three isolates identified as *P. fluorescens*, including one pathogen of *L. edodes* (Komatsu and Goto, 1974). Our methods of comparison included RFLP and partial sequence analysis of PCR-amplified 16S rDNA, and analysis of fingerprints obtained using PCR with primers corresponding to repetitive extragenic palindromic sequences (REP-PCR; Stern et al., 1984; Versalovic et al., 1991). These methods have been used to characterize other bacteria of medical or agricultural significance at levels ranging from species, using 16S sequences and RFLP analysis (Jayarao et al., 1991; Vaneechoutte et al., 1992; Jensen et al., 1993; Hiraishi et al., 1995; Massol-Deya et al., 1995), to strains and pathovars, using REP-PCR (de Bruijn, 1992; Judd et al., 1993; Go et al., 1995; Louws et al., 1995). These methods have also recently been compared for the characterization of strains of *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen (Edel et al., 1995).

Materials and Methods

Strains and molecular techniques The isolates used in this study are listed in Table 1. All of the Tottori Mycological Institute (TMIC) isolates are maintained under liquid nitrogen; isolates used in this study were transferred into 800 μl of Luria broth (Sambrook et al., 1989) in 1.5 ml cryovials, brought to 20% (v/v) glycerol after 24 h growth at 30°C, then maintained at −20°C. All PCR amplifications used cells transferred from these glycerol stock cultures using sterile, desposable 1 μl loops (Fisher Scientific, Pittsburgh, PA). Amplifications of 16S rDNA used primers pA and pH, aligning to positions 8–27 and 1541–1522 on the *Escherichia coli* (Migula) Castellani & Chalmers small ribosomal subunit (Eden et al., 1991; Massol-Deya et al., 1995). Amplifications were carried out in a 100 μl reaction mix under standard conditions (Innis et al., 1990), in a Perkin Elmer 9600 thermocycler (Perkin Elmer, Foster City, CA). Amplifications were assessed by gel electrophoresis, cleaned using Wizard PCR-prep columns (Promega, Madison, WI) and quantified using gel electrophoresis and UV spectrometry before sequence analysis or digestion with restriction endonucleases (Sambrook et al., 1989). Equivalent amounts of PCR products were digested with the 4-base recognition restriction enzymes AluI (Boehringer Mannheim, Indianapolis), *Hae*III, *Msp*I, *Rsa*I, and *Taq*I (BRL, Life Technologies, Gaithersburg, MD), run in 1×TAE–1.5% (w/v) SuperSieve (BRL) gels for 4 h at 3 V/cm, and the gels post-stained with ethidium bromide and photographed over UV light. The primers (REP1R-I and REP2-I) and protocols used for REP-PCR were those described by de Bruijn (1992). Amplifications were carried out in a volume of 25 μl; 8 μl of products were run in 1×TAE-1.5% (w/v) SuperSieve gels for 5 h at 3 V/cm and the gels photographed over UV light. Partial sequences of the 16S amplification products were obtained with primers pA and 519R (Lane et al., 1985), using an automated cycle sequencer and dye-deoxy terminator technology (Applied Biosystems Division, Perkin Elmer).

Data analysis Bands in RFLP and REP-PCR gels were measured and banding patterns compared using a measure of genetic distance (Nei and Li, 1979; Vilgalys and Hester, 1990). Phenograms based on these distances were derived using neighbour-joining clustering (Saitou and Nei, 1987) in the program NTSYS-pc 1.60 (Rohlf, 1991). Sequences recorded by the ABI sequencer from the complementary strands of individual isolates were compared and corrected using SeqEd (ABI software, Perkin Elmer). Similar sequences available in Genbank were searched using BLAST (Altschul et al., 1990). *Acinetobacter* was chosen as a sister group to *Pseudomonas* (Palleroni, 1992), and *E. coli* was chosen as outgroup for the selection of taxa including *Citrobacter* and TMIC 32059 (Table 2). Sequences of these additional taxa were obtained in pre-aligned format from the ribo-

Table 1. Isolates used, their origins, and RFLP, REP-PCR and sequence patterns.

Isolate No.	Identified as	Origin	RFLP group	REP-PCR group	Sequence group	GenBank Accession
TMIC 32228	*Pseudomonas tolaasii*	*Agaricus brunnescens* fruiting body	a	a	a	U63904
TMIC 32238	*P. tolaasii*	*Flammulina velutipes* fruiting body	a	b	a	U63908
TMIC 32237	*P. tolaasii*	*Lentinula edodes* fruiting body	a	c	a	U63907
TMIC 32282	*P. tolaasii*	*Lentinula edodes* fruiting body	a	d	a	U63909
TMIC 32241	*P. fluorescens*	*Lentinula edodes* fruiting body	a	e	a	U63902
TMIC 32061	*P. tolaasii*	*Pleurotus ostreatus* fruiting body	ND[a)]	ND	a	U63903
TMIC 32230	*P. tolaasii*	*Pleurotus ostreatus* fruiting body	a	f	a	U63905
TMIC 32236	*P. tolaasii*	*Pleurotus ostreatus* fruiting body	a	f	a	U63906
IFO 3507	*P. fluorescens*	Creamery waste	b	g	b	U63901
TMIC 32059	*P. fluorescens* biovar. B	Wood colonized by *Pleurotus pulmonarius*	c	h	c	U63900
IFO 14595	*Burkholderia cepacia*	Unknown (phytopathogenic)	ND	i	ND	ND

a) Not determined.

Table 2. Sequences used, their origins and sequence patterns.

Isolate No.	Identified as	Origin	Sequence group	GenBank Accession
ATCC 17925	*Acinetobacter lwoffii*	Unknown	d	U10875
DSM 1727	*Azospirillum* sp.	Plant litter	b[a)]	Z29622
ATCC 29935	*Citrobacter freundii*	Livestock	e	M59291
MG 1655	*Escherichia coli* K-12	Human patient	f	L10328
DSM 50071	*P. aeruginosa*^{T}	Unknown	g	X06684
NCPPB 3063	*P. flavescens*^{T}	Canker on *Juglans*	h	U01916
MS 1650	*P. fluorescens* 2	Unknown	i	RDP[b)]
ATCC 27951	"*Flavobacterium lutescens*"	Yoghurt	j	M59156
ATCC 25411	*P. mendocina*	Ethanol-enriched soil	k	M59154
NCIB 453512	*P. putida* PaWI	Soil (BTEX-degrading)	l	L28676
A 501	*P. syringae*	Unknown	m	RDP

a) 100% identity with region sequenced from IFO 3507 in this study.
b) Ribosomal Database Project (Maidak et al., 1994), source E. R. B. Moore, unpublished.

somal database project (RDP, Maidak et al., 1994) via Netscape Navigator 1.1 (Netscape Communications, Mountain View, CA). Phylogenetic analysis of thirteen unique sequences was made using PAUP 3.0s (Swofford, 1991). Phenetic analysis including all isolates sequenced in this study was made using the mean genetic distances provided in PAUP, and UPGMA clustering in NTSYS-pc.

Results

RFLP analyses Using whole cells from glycerol stocks as templates, PCR amplification products of 16S rDNA were obtained from all isolates except TMIC 32061. For this isolate, 16S rDNA was amplified from genomic DNA isolated from a stock culture (Sambrook et al., 1989). Identical banding patterns were obtained in restriction digests with all enzymes of all *P. tolaasii* isolates and *P. fluorescens* TMIC 32241, a mushroom pathogen (Komatsu and Goto, 1974) (Fig. 1). Non-pathogenic *P. fluorescens* IFO 3507 differed only with *Msp*I. In con-

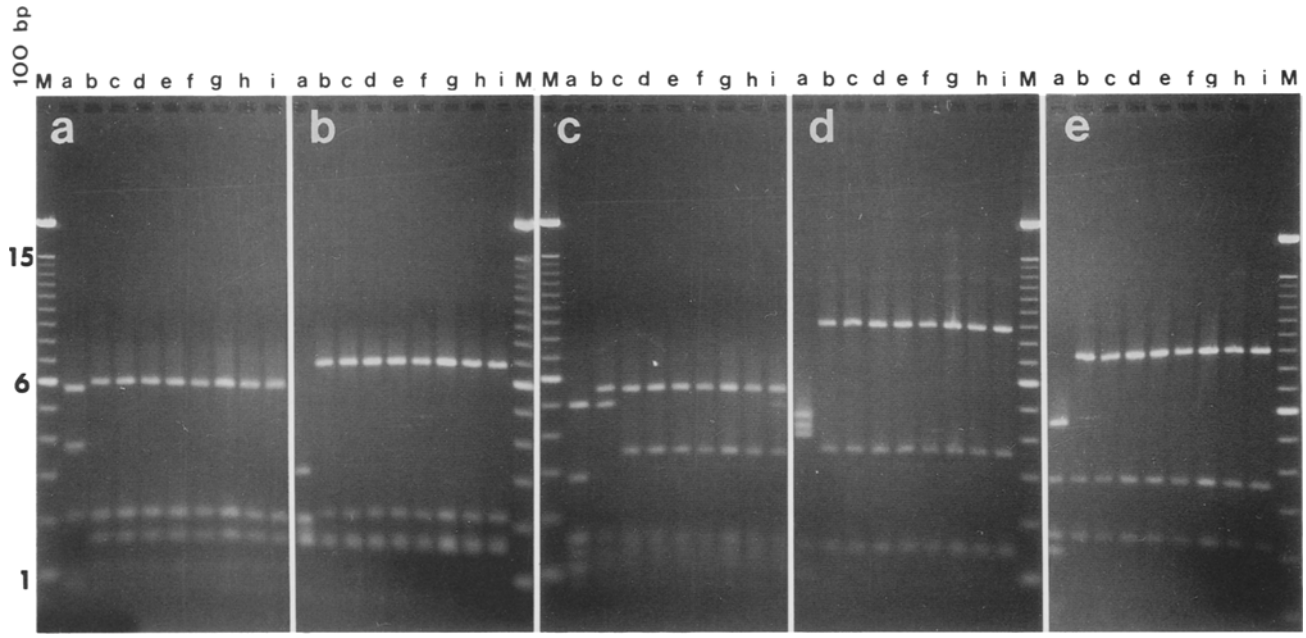

Fig. 1. Banding patterns of PCR-amplified 16S rDNA from various *Pseudomonas* species, digested with five different restriction endonucleases.
Photographs: a. AluI. b. HaeIII. c. MspI. d. RsaI. e. TaqI. Size markers (M) are 100 base-pair ladders. Lanes: a. TMIC 32059; b. IFO 3507; c. TMIC 32241; d. TMIC 32228; e. TMIC 32230; f. TMIC 32236; g. TMIC 32237; h. TMIC 32238; i. TMIC 32282.

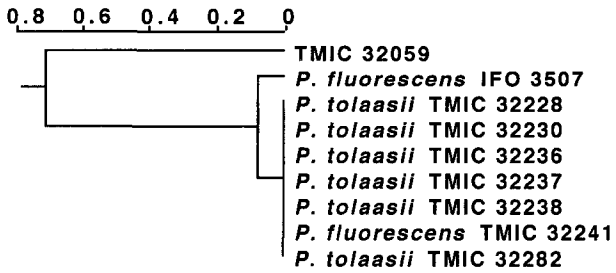

Fig. 2. Phenogram using the neighbour-joining method of clustering, based on RFLP banding patterns shown in Fig. 1. Cophenetic correlation coefficient (r) = 0.999.

trast, the digests of "*P. fluorescens*" TMIC 32059 were markedly different with all enzymes. A phenogram based on distances calculated from a measure of "nucleotide diversity" (Nei and Li, 1979) graphically illustrates these results (Fig. 2).

Analysis of partial 16S sequences Sequences derived from PCR-amplified 16S rDNA using primers pA and 519R gave complementary sequences with approximately 550 and 490 readable bases, respectively, of which 486 showed clear overlap for base confirmation. Corrected sequences differed by 2.3–13.8% from the machine-read originals (data not shown). All isolates of *P. tolaasii* and the mushroom pathogen identified as *P. fluorescens* TMIC 32241 yielded identical sequences in this region. Searches using BLAST for sequences available in Genbank similar to *P. tolaasii* yielded "*Azospirillum* sp." DSM 1727, *P. putida* (Trevisan) Migula, *P. flavescens* Hildebrand, *P. mendocina* Palleroni, *P. aeruginosa* (Schroeter) Migula, and "*Flavobacterium lutescens*" in the top 10 sequences. The sequence of non-pathogenic *P. fluorescens* IFO 3507 is identical to the sequence of "*Azospirillum* sp." DSM 1727 and differed from those of *P. tolaasii* by only 4 bases (0.8%), with no gaps. The sequence of TMIC 32059, identified as *P. fluorescens* from wood colonized by *Pleurotus pulmonarius* (Fr.) Quélet (Thorn and Tsuneda, 1992), differed by 43 bases (8%) and required 9 gaps for alignment to *P. tolaasii* sequences. A BLAST search using this sequence yielded *Citrobacter freundii* (Braak) Werkman & Gillen) as the closest match in Genbank, differing by 12 bases (2.4%). Contamination at some stage between identification of the strain and isolation and sequencing of the DNA cannot be ruled out. A cladogram based on these sequences is shown in Fig. 3. In this cladgram, *P. tolaasii* is placed within a subgroup of *Pseudomonas* that includes *P. fluorescens* and *P. syringae*, and which is a sister group to a second set including *P. flavescens*, *P. mendocina*, *P. aeruginosa* (the type species of *Pseudomonas*), *P. putida*, and "*Flavobacterium lutescens*." This cladogram links the sequence from TMIC 32059 ("*P. fluorescens*") with that of *C. freundii* (Enterobacteria-

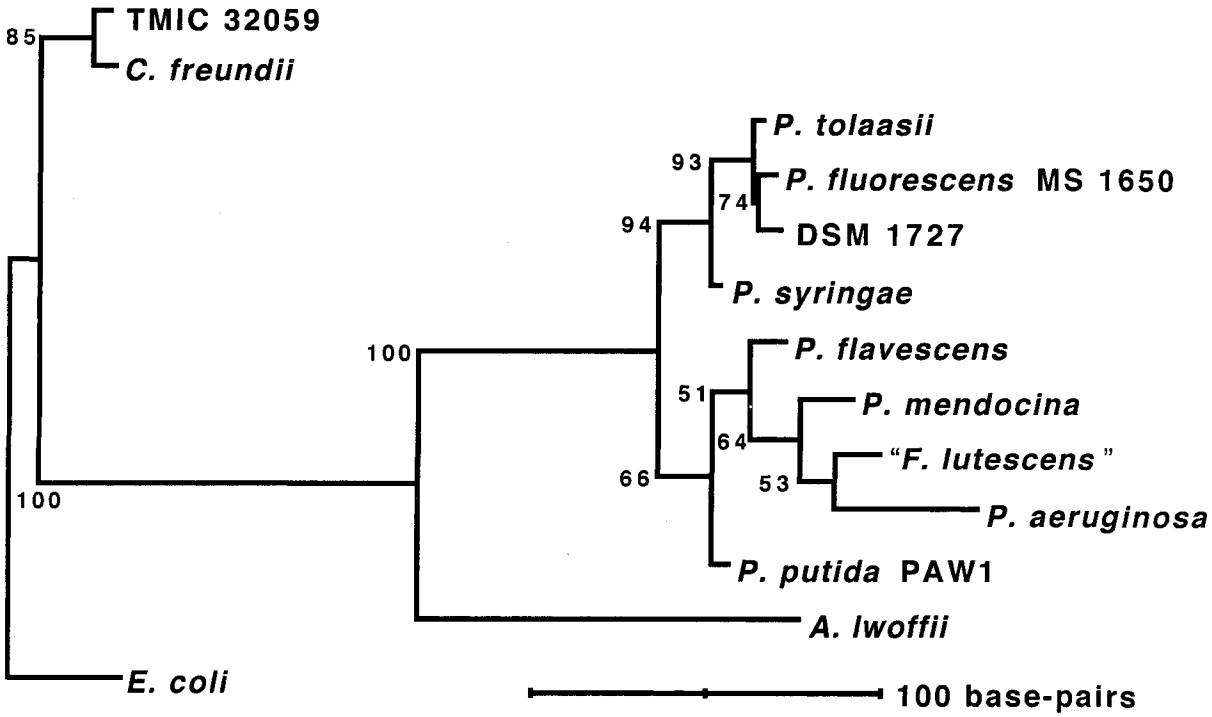

Fig. 3. Cladogram using the maximum parsimony method based on partial 16S nucleotide sequences from *Pseudomonas* isolates obtained in this study and from Genbank and RDP (see Tables 1 and 2).
Bootstrap confidence values (n = 100) are shown at branch bases. This is an unrooted tree, but has been drawn as rooted with *E. coli* and the remainder of taxa monophyletic. Branch lengths are proportional to genetic distance. The branch containing all isolates of *P. tolaasii* also includes *P.* "*fluorescens*" (TMIC 32241), and the branch labelled DSM 1727 also contains *P. fluorescens* (IFO 3507).

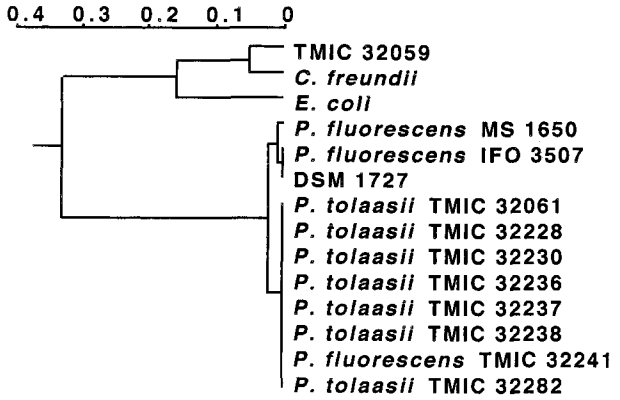

Fig. 4. Phenogram using the UPGMA method of clustering, based on genetic distances calculated from partial 16S nucleotide sequences.
Cophenetic correlation coefficient (r)=0.935

ceae), and that of DSM 1727 ("*Azospirillum*" sp.) with *P. fluorescens*.

Figure 4 shows a phenogram that includes all *P. tolaasii* and *P. fluorescens* isolates plus *C. freundii* and *E. coli*. As in RFLP analyses (Fig. 2), all isolates of *P.*

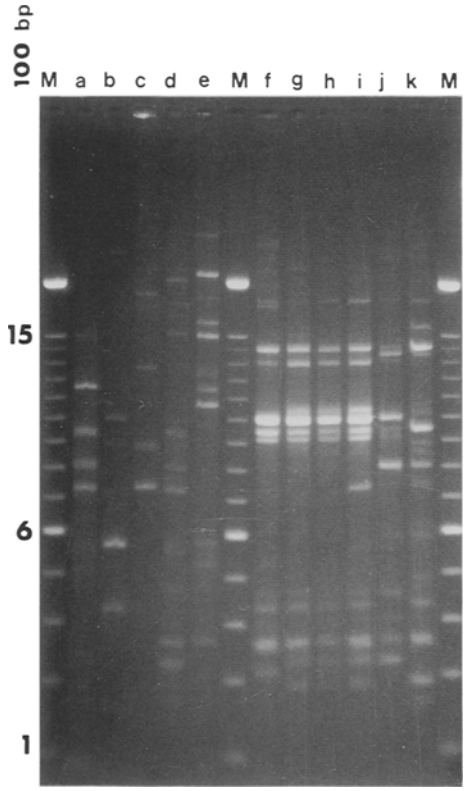

Fig. 5. Banding pattern of REP-PCR of various *Pseudomonas* isolates. Size markers are 100 base-pair ladders (M).
Lanes a. TMIC 32059; b. *P. putida* TMIC 3738; c. IFO 3507; d. TMIC 32241; e. IFO 14595; f. TMIC 32228; g. TMIC 32230; h. TMIC 32236; i. TMIC 32238; j. TMIC 32282; k. TMIC 32237.

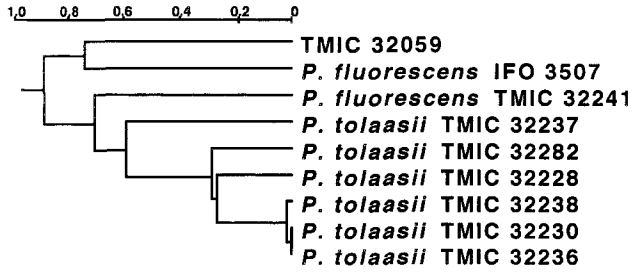

Fig. 6. Phenogram using the neighbour-joining method of clustering, based on REP-PCR banding patterns shown in Fig. 5. Cophenetic correlation coefficient (r)=0.966.

tolaasii plus the mushroom-pathogenic *P. fluorescens* are identical, and these are most similar to a group of *P. fluorescens* including IFO 3507, MS 1650, and DSM 1727. Isolate TMIC 32059 is grouped with *C. freundii* on a branch with *E. coli*.

Analysis of REP-PCR REP-PCR yielded 4 bands in IFO 3507, 5 in TMIC 32059, and 10–12 bands in isolates of *P. tolaasii* (Fig. 5). Among *P. tolaasii* isolates, TMIC 32228, 32230, 32236, and 32238 shared very similar banding patterns (d=0–0.3), and of these, 32230 and 32236, both pathogens of *Pleurotus ostreatus* but from different parts of Japan, were identical. The patterns of 32237, 32241 and 32282, pathogens of *Lentinula edodes*, were most dissimilar to each other and to other *P. tolaasii* isolates (d=0.27–0.80), but each shared several prominent bands with other *P. tolaasii* isolates. Isolate IFO 3507, the non-pathogenic *P. fluorescens*, shared only one band with each of TMIC 32059 and TMIC 32238, but had none in common with other isolates of *P. tolaasii*.

Figure 6 shows a phenogram based on the genetic distances indicated by the REP-PCR patterns of these isolates. The two isolaes from *P. ostreatus* were identical, and most similar to strains from *F. velutipes* and *A. brunnescens*, and these are joined at distances from 0.4 to 0.7 with the isolates from *L. edodes*. TMIC 32059 and IFO 3507 are joined on a branch separate from *P. tolaasii*, but implication of their connection from this tree, despite its good statistical support, should be viewed with skepticism because of the branching deph (Rohlf, 1991).

Discussion

Bergey's Manual of Systematic Bacteriology (Palleroni, 1984) and Young et al. (1978) recognize *P. tolaasii* as a species distinct from *P. fluorescens*. de Vos et al. (1985) showed with DNA:23S rRNA hybridization that *P. tolaasii* belongs in "true *Pseudomonas*" (the *P. fluorescens* group), since the per cent hybridization of *P. tolaasii* with *P. fluorescens* was the same as with *P. fluorescens* hybridized with itself. In their similarity map of estimated melting temperatures [Tm(e)] versus % rRNA binding, *P. tolaasii* strains formed a cluster that was within the *P. fluorescens* group, and was closest to the type strain (ATCC 13525) of *P. fluorescens*. These authors point out that their method does not allow sepa-

ration of species within an rRNA branch and suggested that "our findings should induce extensive experimentation on the relationships among these species and pathovars" (de Vos et al., 1985). The data from RFLP and REP-PCR banding patterns and partial 16S rDNA sequences in our study suggest that *P. tolaasii* and *P. fluorescens* IFO 3507 represent distinct species, although the two are unquestionably very closely related. The nearly complete differences in REP-PCR banding patterns between IFO 3507 and isolates of *P. tolaasii* are characteristic of interspecific comparisons using REP-PCR (Louws et al., 1995). These patterns, plus the consistent sequences and RFLP patterns of *P. tolaasii* isolates and the slight differences in these from those of IFO 3507, show that *P. tolaasii* and *P. fluorescens* can be distinguished genetically, but are insufficient to draw conclusions as to taxonomic rank. DNA: DNA hybridization experiments using total genomic DNA from more isolates, including the type strains of *P. tolaasii* and *P. fluorescens*, will be required to determine the status of these two taxa (Wayne et al., 1987; Fox et al., 1992; Stackebrandt and Goebel, 1994). DNA reassociation offers a more powerful tool than sequencing of 16S rDNA to recognize recently diverged bacterial species (Fox et al., 1992). The phylogenetic definition of bacterial species includes strains with greater than 70% DNA-DNA relatedness and 5°C or less ΔTm (Wayne et al., 1987). Several examples exist of species with complete 16S sequences differing by less than 1% but with less than 50% DNA-DNA reassociation (Fox et al., 1992).

The only other published gene sequence data relating to *P. tolaasii* and *P. fluorescens* is of the gene encoding the major outer-membrane protein OprF (de Mot et al., 1994). As with our results using partial sequences of 16S rRNA genes, the sequence of oprF genes in *P. tolaasii* and *P. fluorescens* indicated that these two species are closely related. Indeed, their dendrogram based on oprF sequences, which also includes *P. aeruginosa* and *P. syringae*, has same topology as our Fig. 3: *P. tolaasii* and one strain identified as *P. fluorescens* are slightly basal to five other *P. fluorescens* isolates, and *P. aeruginosa* and *P. syringae* are on a separate branch.

Using physiological tests including carbon assimilation, reduction of nitrate, sensitivity to sodium chloride, and electrophoresis of soluble cellular proteins, Goor et al. (1986) found that strains of *P. tolaasii* formed a tight cluster similar to but separable from *P. aeruginosa*, *P. fluorescens* biovar II, white line reacting organisms ("*P. reactans*"), and the mushroom pathogens "*P. gingeri*," *P. agarici* Young, and isolates causing mummy disease. Likewise, in this preliminary study, the RFLP patterns and partial sequences of PCR-amplified 16S rDNA of all strains of *P. tolaasii* and one mushroom pathogen previously identified as *P. fluorescens* are idential, but differ slightly from those of non-mushroom-pathogenic *P. fluorescens*. Thus RFLP analysis of amplified rDNA may prove a useful technique for the rapid identification of the diversity of pseudomonads that are associated with mushrooms (Preece, 1988). Greater variability is present in the large subunit (23S; Christensen et al., 1994) and the spacer region between 16S and 23S subunits (Gill et al., 1994) than in the small (16S) subunit which was amplified in our study. Restriction digests of a larger amplified fragment including the small and large subunits and intervening spacer should improve the sensitivity of this technique (Vaneechoutte et al., 1992; Jensen et al., 1993; Massol-Deya et al., 1995).

Previous studies by Goor et al. (1986), using physiological characters and protein electrophoresis, established the homogeneity and distinctness of *P. tolaasii*, but were unable to separate pathogenic from non-pathogenic strains, strains from different hosts, and strains that did not give white line reactions. Using REP-PCR, we found differences among *P. tolaasii* isolates originating from different mushroom hosts, and which had been shown to differ in their pathogenicity to *L. edodes* (Tsuneda and Thorn, 1994). REP-PCR also yielded markedly different fingerprints for specifically distinct isolates such as the non-pathogen *P. fluorescens* IFO 3507 and mushroom-pathogenic *Burkholderia* (*Pseudomonas*) *cepacia* (Palleroni & Holmes) Yabuuchi et al. (Yabuuchi et al., 1992) (Fig. 5). This technique provides a fast and efficient means of characterizing bacterial isolates at the strain (subspecific) level (Versalovic et al., 1992); 96 isolates could potentially be compared in 10 h (6 h for PCR amplification, and 3–4 h for gel electrophoresis of the PCR products). Although it was not required in this study, sensitivity of this technique could also be increased by additional amplifications using primers corresponding to enterobacterial repetitive intergeneric consensus (ERIC) and BOX element sequences (Louws et al., 1995). A much larger survey of *P. tolaasii* isolates, including strains that are non-pathogenic, pathogenic on different mushroom hosts, and from a wide geographical background, is desirable. These studies may yield valuable insights into the distribution and origin(s) of *P. tolaasii*, as well as its pathogenicity, host specificity, and susceptibility to bacteriolysis by wood decay fungi cultivated as commercial mushroom crops.

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