Detection of the outer membrane lipoprotein I and its gene in fluorescent and non-fluorescent pseudomonads: implications for taxonomy and diagnosis

D. DE VOS,¹ A. LIM, JR,¹ P. DE VOS,² A. SARNIGUET,³ K. KERSTERS² and P. CORNELIS^{1*}

¹Laboratorium Algemene Biologie, Vrije Universiteit Brussel, Paardenstraat 65, B-1640 St-Genesius Rode, Belgium

²Laboratorium voor Microbiologie, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

³INRA, Centre de Recherche de Rennes, Station de Pathologie Végétale, B.P. 26, 35650 Le Rheu, France

(Received 11 November 1992; revised 31 March 1993; accepted 2 April 1993)

The open reading frame of the *OprI* lipoprotein gene from *Pseudomonas aeruginosa* was amplified by polymerase chain reaction (PCR) starting from purified DNA or colony lysates. A fragment of the expected size (249 bp) was detected in all *P. aeruginosa* strains from various clinical and geographical origins. The gene could only be amplified in pseudomonads of rRNA group I which are considered to be the authentic genus *Pseudomonas*. Digestions with *HaeIII*, *PvuII* and *SphI* of the amplified fragments demonstrated a sequence variation in the *oprI* gene. Colony, dot and Western blots with two monoclonal antibodies (mAbs) against the lipoprotein I confirmed our PCR results. These findings open interesting perspectives for the molecular taxonomy of the genus *Pseudomonas* and the development of diagnostic tools.

Introduction

Pseudomonas aeruginosa is a typical opportunistic pathogen (Botzenhart & Rüden, 1987). It is more and more frequently recognized as a causative agent of a variety of mostly complicated and often fatal infections (Bodey *et al.*, 1983; Mondino *et al.*, 1986), including septicaemia (Bodey *et al.*, 1985) and lung infections in patients with cystic fibrosis (Govan, 1986; Hoiby *et al.*, 1987). In view of the increasing clinical importance of *P. aeruginosa*, the development of a rapid and specific diagnostic test based on the polymerase chain reaction (PCR) and/or monoclonal antibody (mAb) detection could contribute to a better management of this clinical pathogen. According to Saint-Onge *et al.* (1992) the *oprI* gene is a generally applicable PCR target region for the *Pseudomonas* species of rRNA group I (Palleroni, 1984), which is at present considered to be the authentic genus *Pseudomonas* (De Vos & De Ley, 1983). However, in addition to the rRNA group I *Pseudomonas* species (Palleroni, 1984) which were partly included by Saint-Onge *et al.* (1992), many more phytopathogenic and saprophytic species which have the name *Pseudomonas* belong to this phylogenetic group (De Vos *et al.*, 1985, 1989). Therefore

we extended the experiments of Saint-Onge *et al.* (1992) by including other *Pseudomonas* rRNA group I members as well as the free-living nitrogen-fixing *Azotobacter* and *Azomonas* species which are also located in the same phylogenetic neighbourhood as the authentic pseudomonads (De Vos & De Ley, 1983).

The question then arises whether the *oprI* amplification can be used for a specific detection of *Pseudomonas aeruginosa* strains or of other important fluorescent pseudomonads acting as plant-growth-promoting rhizobacteria (Davison, 1988; Weller, 1988). We compared the *PvuII*, *HaeIII* and *SphI* restriction patterns of the amplicates of the different organisms in order to find a species-specific profile for *P. aeruginosa* and closely-related organisms. This knowledge will not only enhance the development of a specific diagnostic test but will also provide complementary genotypic data for the classification of the *Pseudomonas* species from rRNA group I at species and subspecies level. At present, the finer classification of this important group of micro-organisms is not well defined and identifications are often not reliable.

Methods

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. Strains not obtained from culture collections were identified according to classical bacteriological procedures. The bacteria were grown overnight in 3 ml Luria-Bertani (LB) broth

* Author for correspondence. Tel. 2 3590258; fax 2 3590390.

Abbreviations: mAb, monoclonal antibody; RFLP, restriction fragment length polymorphism.

Table 1. *Bacterial species used in this study*

The *Pseudomonas* species outside rRNA group I (Palleroni, 1984) are misclassified and the genus name is therefore mentioned in brackets.

Species	Strain*	Geographical origin	Source	Provided by†
<i>Pseudomonas aeruginosa</i>	PAO 1	France	Clinical isolate, wild type	J. M. Meyer, ULP, Strasbourg
<i>P. aeruginosa</i>	PA 3	Belgium	Clinical isolate, cystic fibrosis	G. Wauters, UCL, Brussels
<i>P. aeruginosa</i>	PA 4	Belgium	Clinical isolate, urinary infection	G. Wauters, UCL, Brussels
<i>P. aeruginosa</i>	PA 5	Belgium	Clinical isolate, urinary infection	G. Wauters, UCL, Brussels
<i>P. aeruginosa</i>	PA 6	Belgium	Clinical isolate, Intensive Care unit	G. Wauters, UCL, Brussels
<i>P. aeruginosa</i>	PA 7	Belgium	Clinical isolate, urinary infection	G. Wauters, UCL, Brussels
<i>P. aeruginosa</i>	PA 8	Belgium	Clinical isolate, urinary infection	G. Wauters, UCL, Brussels
<i>P. aeruginosa</i>	PA 10	Belgium	Clinical isolate, urinary infection	G. Wauters, UCL, Brussels
<i>P. aeruginosa</i>	PA 11	Belgium	Clinical isolate, urinary infection	G. Wauters, UCL, Brussels
<i>P. aeruginosa</i>	PA 12	Belgium	Clinical isolate, urinary infection	G. Wauters, UCL, Brussels
<i>P. aeruginosa</i>	PA 1022	Belgium	Clinical isolate	H. Goossens, U.Z. Sint Pieters, Brussels
<i>P. aeruginosa</i>	PA 1601	Belgium	Clinical isolate	H. Goossens, U.Z. Sint Pieters, Brussels
<i>P. aeruginosa</i>	PA 2001	Belgium	Clinical isolate	H. Goossens, U.Z. Sint Pieters, Brussels
<i>P. aeruginosa</i>	PA 2019	Belgium	Clinical isolate	H. Goossens, U.Z. Sint Pieters, Brussels
<i>P. aeruginosa</i>	MH 1	Belgium	Clinical isolate, burn wound	M. Vanderkelen, Mil. Hosp., Brussels
<i>P. aeruginosa</i>	MH 2	Belgium	Clinical isolate, burn wound	M. Vanderkelen, Mil. Hosp., Brussels
<i>P. aeruginosa</i>	MH 3	Belgium	Clinical isolate, burn wound	M. Vanderkelen, Mil. Hosp., Brussels
<i>P. aeruginosa</i>	MH 4	Belgium	Clinical isolate, burn wound	M. Vanderkelen, Mil. Hosp., Brussels
<i>P. aeruginosa</i>	MH 6	Belgium	Clinical isolate, burn wound	M. Vanderkelen, Mil. Hosp., Brussels
<i>P. aeruginosa</i>	MH 7	Belgium	Clinical isolate, burn wound	M. Vanderkelen, Mil. Hosp., Brussels
<i>P. aeruginosa</i>	MH 8	Belgium	Clinical isolate, burn wound	M. Vanderkelen, Mil. Hosp., Brussels
<i>P. aeruginosa</i>	MH 9	Belgium	Clinical isolate, burn wound	M. Vanderkelen, Mil. Hosp., Brussels
<i>P. aeruginosa</i>	MH 10	Belgium	Clinical isolate, burn wound	M. Vanderkelen, Mil. Hosp., Brussels
<i>P. aeruginosa</i>	DW 1	Philippines	Clinical isolate, urinary infection	F. de la Peña, R. de los Reyes, DWU, Tacloban
<i>P. aeruginosa</i>	DW 2	Philippines	Clinical isolate, bacteraemia	F. de la Peña, R. de los Reyes, DWU, Tacloban
<i>P. aeruginosa</i>	DW 3	Philippines	Clinical isolate, wound infection	F. de la Peña, R. de los Reyes, DWU, Tacloban
<i>P. aeruginosa</i>	DW 4	Philippines	Clinical isolate, wound infection	F. de la Peña, R. de los Reyes, DWU, Tacloban
<i>P. aeruginosa</i>	DW 5	Philippines	Clinical isolate, urinary infection	F. de la Peña, R. de los Reyes, DWU, Tacloban
<i>P. aeruginosa</i>	DW 6	Philippines	Clinical isolate, post operative infection	F. de la Peña, R. de los Reyes, DWU, Tacloban
<i>P. aeruginosa</i>	PS 3707	Australia	Clinical isolate, cystic fibrosis	B. Holloway, Monash University
<i>P. aeruginosa</i>	PS 3880	Australia	Clinical isolate, cystic fibrosis	B. Holloway, Monash University
<i>P. aeruginosa</i>	PS 3879	Australia	Clinical isolate, cystic fibrosis	B. Holloway, Monash University
<i>P. aeruginosa</i>	PS 3883	Australia	Clinical isolate, cystic fibrosis	B. Holloway, Monash University
<i>P. aeruginosa</i>	PS 3711	Australia	Clinical isolate, cystic fibrosis	B. Holloway, Monash University
<i>P. aeruginosa</i>	7NSK2	Belgium	Soil isolate	M. Höfte, RUG, Ghent
<i>P. agarici</i>	LMG 2113	New Zealand	Cultivated mushroom isolate	LMG
<i>P. agarici</i>	LMG 2112 ^T	New Zealand	Cultivated mushroom isolate	LMG
<i>P. agarici</i>	LMG 2110	United Kingdom	Cultivated mushroom isolate	LMG
<i>P. alcaligenes</i>	LMG 1224 ^T	USA	Water isolate, swimming pool	LMG
<i>P. asplenii</i>	LMG 2137 ^T	ND	Plant isolate, <i>Asplenium nidus</i>	LMG
<i>P. aureofaciens</i>	LMG 1245 ^T	The Netherlands	River clay, suspended in kerosene	LMG
<i>P. chlororaphis</i>	T 9	Belgium	Rhizosphere isolate, <i>Lycopersicon lycopersicum</i>	P. Thonart, ULg, Liege
<i>P. cichorii</i>	LMG 2162 ^T	Germany	Plant isolate, <i>Cichorii endiva</i>	LMG
<i>P. corrugata</i>	LMG 2172 ^T	United Kingdom	Plant isolate, <i>Lycopersicon lycopersicum</i>	LMG
<i>P. fluorescens</i>	ATCC 17400	USA	Poultry industry, hen's egg	J. M. Meyer, ULP, Strasbourg
<i>P. fluorescens</i>	AS 1	France	Wheat rhizosphere isolate	A. Sarniguet, INRA, Rennes
<i>P. fluorescens</i>	AS 11	France	Wheat rhizosphere isolate	A. Sarniguet, INRA, Rennes

Table 1. (cont.)

Species	Strain*	Geographical origin	Source	Provided by†
<i>P. fluorescens</i>	AS 14	France	Wheat rhizosphere isolate	A. Sarniguet, INRA, Rennes
<i>P. fluorescens</i>	AS 21	France	Wheat rhizosphere isolate	A. Sarniguet, INRA, Rennes
<i>P. fluorescens</i>	AS 22	France	Wheat rhizosphere isolate	A. Sarniguet, INRA, Rennes
<i>P. fluorescens</i>	AS 25	France	Wheat rhizosphere isolate	A. Sarniguet, INRA, Rennes
<i>P. fluorescens</i>	AS 30	France	Wheat rhizosphere isolate	A. Sarniguet, INRA, Rennes
<i>P. fluorescens</i>	AS 35	France	Wheat rhizosphere isolate	A. Sarniguet, INRA, Rennes
<i>P. fluorescens</i>	AS 37	France	Wheat rhizosphere isolate	A. Sarniguet, INRA, Rennes
<i>P. fluorescens</i>	AS 39	France	Wheat rhizosphere isolate	A. Sarniguet, INRA, Rennes
<i>P. fluorescens</i>	AS 46	France	Wheat rhizosphere isolate	A. Sarniguet, INRA, Rennes
<i>P. fluorescens</i>	AS 48	France	Wheat rhizosphere isolate	A. Sarniguet, INRA, Rennes
<i>P. fragi</i>	LMG 2191 ^T	ND	ND	LMG
<i>P. fuscovagiae</i>	LMG 2158 ^T	Japan	Plant isolate, <i>Oryza sativa</i>	LMG
<i>P. marginalis</i>	LMG 2238	USA	Plant isolate, <i>Medicago sativa</i>	LMG
<i>P. marginalis</i>	LMG 2214	USA		LMG
<i>P. mendocina</i>	LMG 1223 ^T	Argentina	Soil isolate, soil enrichment	LMG
<i>P. oleovorans</i>	LMG 2229 ^T	USA	Oil/water suspension	LMG
<i>P. pseudoalcaligenes</i>	LMG 1225 ^T	USA	Clinical isolate, sinus discharge	LMG
<i>P. putida</i> biovar A	LMG 2257 ^T	USA	Soil isolate, lactate enrichment	LMG
<i>P. putida</i> biovar B	LMG 1246	ND	ND	LMG
<i>P. reptilovora</i>	LMG 2273 ^T	ND	ND	LMG
<i>P. stutzeri</i>	LMG 2333 ^T	USA	Clinical isolate, spinal fluid	LMG
<i>P. syringae</i>	LMG 1247	UK	Plant isolate, <i>Syringa vulgaris</i>	LMG
<i>P. taetrolens</i>	LMG 2336 ^T	ND	ND	LMG
<i>P. tolaasii</i>	LMG 2340	UK	Cultivated mushroom isolate	LMG
<i>P. tolaasii</i>	LMG 2342 ^T	UK	Cultivated mushroom isolate	LMG
(<i>P.</i>) <i>andropogonis</i>	LMG 2129 ^T	USA	Plant isolate, <i>Sorghum bicolor</i>	LMG
(<i>P.</i>) <i>cepacia</i>	LMG 6981	ND	Clinical isolate, bronchial washing	LMG
(<i>P.</i>) <i>diminuta</i>	LMG 1793 ^T	USA	Water isolate, fresh water river	LMG
(<i>P.</i>) <i>gladioli</i>	LMG 2216	USA	Plant isolate, <i>Gladiolus</i> sp.	LMG
(<i>P.</i>) <i>glumae</i>	LMG 2196 ^T	Japan	Plant isolate, <i>Oryza sativa</i>	LMG
(<i>P.</i>) <i>huttienensis</i>	LMG 2199 ^T	New Zealand	Isolate from distilled water	LMG
(<i>P.</i>) <i>pickettii</i>	LMG 6866	ND	ND	LMG
(<i>P.</i>) <i>pickettii</i>	LMG 7056	Belgium	Clinical isolate	LMG
(<i>P.</i>) <i>pickettii</i>	LMG 5942 ^T	USA	Clinical isolate	LMG
(<i>P.</i>) <i>pickettii</i>	LMG 6871	Senegal	Rice rhizosphere isolate	LMG
(<i>P.</i>) <i>solanacearum</i>	LMG 2299 ^T	USA	Plant isolate, <i>Lycopersicon lycopersicum</i>	LMG
(<i>P.</i>) <i>solanacearum</i>	LMG 2296	Zimbabwe	Plant isolate, <i>Lycopersicon lycopersicum</i>	LMG
(<i>P.</i>) <i>solanacearum</i>	LMG 2297	Mauritius Islands	Plant isolate, <i>Casuarina equisetifolia</i>	LMG
(<i>P.</i>) <i>solanacearum</i>	LMG 2306	Portugal	Plant isolate, <i>Lycopersicon lycopersicum</i>	LMG
(<i>P.</i>) <i>vesicularis</i>	LMG 2350 ^T	Belgium	Leech	LMG
(<i>P.</i>) <i>woodsii</i>	LMG 2362 ^T	USA	Plant isolate, <i>Dianthus caryophyllus</i>	LMG
<i>Acinetobacter</i> sp.	GC 2	Belgium	Clinical isolate	G. Cornelis, UCL, Brussels
<i>Alcaligenes eutrophus</i>	CH 34	Belgium	Soil isolate	M. Mergeay, SCK-VITO, Mol
<i>Azomonas macrocytogenes</i>	LMG 8755 ^T	Denmark	Soil isolate	LMG
<i>Azomonas macrocytogenes</i>	LMG 1266	Denmark	Soil isolate	LMG
<i>Azotobacter paspali</i>	LMG 3864 ^T	Brazil	Soil isolate	LMG
<i>Azotobacter vinelandii</i>	LMG 3884	CIS, Ukraine Republik	Water isolate, Black Sea	LMG
<i>Chryseomonas luteola</i>	LMG 7041 ^T	ND	Clinical isolate	LMG
<i>Comamonas acidovorans</i>	LMG 1226 ^T	The Netherlands	Soil enrichment isolate, acetamide	LMG
<i>Escherichia coli</i>	MM 294	Belgium	Lab strain	P. Cornelis, VUB, Brussels
<i>Klebsiella oxytoca</i>	GC 3	Belgium	Clinical isolate	G. Cornelis, UCL, Brussels
<i>Levinea</i> sp.	GC 1	Belgium	Clinical isolate	G. Cornelis, UCL, Brussels
<i>Proteus vulgaris</i>	GC 5	Belgium	Clinical isolate	G. Cornelis, UCL, Brussels
<i>Providencia stuartii</i>	GC 6	Belgium	Clinical isolate	G. Cornelis, UCL, Brussels
<i>Salmonella typhimurium</i>	GC 4	Belgium	Clinical isolate	G. Cornelis, UCL, Brussels
<i>Serratia marcescens</i>	GC 7	Belgium	Clinical isolate	G. Cornelis, UCL, Brussels
<i>Sphingomonas paucimobilis</i>	LMG 1227 ^T	United Kingdom	Hospital respirator	LMG
<i>Xanthomonas campestris</i>	LMG 568 ^T	United Kingdom	Plant isolate, <i>Brassica</i> sp.	LMG
<i>Xanthomonas maltophilia</i>	LMG 958 ^T	Belgium	Clinical isolate	LMG
<i>Xanthomonas maltophilia</i>	HSP 1	Belgium	Clinical isolate	H. Goossens, U.Z. St Pieters, Brussels
<i>Xanthomonas maltophilia</i>	HSP 34	Belgium	Clinical isolate	H. Goossens, U.Z. St Pieters, Brussels

ND, Not determined.

*T, Type strain.

† LMG, culture collection, Laboratorium voor Microbiologie, University of Ghent, Ghent Belgium; ATCC, American Type Culture Collection, Rockville, Md, USA.

Table 2. Results for PCR and dot blot tests with mAb 3A9 for the presence of lipoprotein I in *Pseudomonas* spp. and other Gram-negative organisms

Species positive in PCR and dot blot		Species negative in PCR and dot blot	
Species	Number tested	Species	Number tested
<i>Pseudomonas aeruginosa</i>	35	(<i>P.</i>) <i>andropogonis</i>	1
<i>P. agarici</i>	3	(<i>P.</i>) <i>cepaia</i>	1
<i>P. alcaligenes</i>	1	(<i>P.</i>) <i>diminuta</i>	1
<i>P. asplenii</i>	1	(<i>P.</i>) <i>gladioli</i>	1
<i>P. aureofaciens</i>	1	(<i>P.</i>) <i>glumae</i>	1
<i>P. chlororaphis</i>	1	(<i>P.</i>) <i>huttiensis</i>	1
<i>P. cichorii</i>	1	(<i>P.</i>) <i>pickettii</i>	4
<i>P. corrugata</i>	1	(<i>P.</i>) <i>solanacearum</i>	4
<i>P. fluorescens</i>	13	(<i>P.</i>) <i>vesicularis</i>	1
<i>P. fragi</i>	1	(<i>P.</i>) <i>woodsii</i>	1
<i>P. fuscovaginae</i>	1	<i>Acinetobacter</i> sp.	1
<i>P. marginalis</i>	2	<i>Alcaligenes eutrophus</i>	1
<i>P. mendocina</i>	1	<i>Azomonas macrocytogenes</i>	2
<i>P. oleovorans</i>	1	<i>Azotobacter paspali</i>	1
<i>P. pseudoalcaligenes</i>	1	<i>Azotobacter vinelandii</i>	1
<i>P. putida</i>	2	<i>Chryseomonas luteola</i>	1
<i>P. reptilovora</i>	1	<i>Comamonas acidovorans</i>	1
<i>P. stutzeri</i>	1	<i>Escherichia coli</i>	1
<i>P. syringae</i>	1	<i>Klebsiella oxytoca</i>	1
<i>P. taetrolens</i>	1	<i>Levinia</i> sp.	1
<i>P. tolaasii</i>	2	<i>Proteus vulgaris</i>	1
		<i>Providencia stuartii</i>	1
		<i>Salmonella typhimurium</i>	1
		<i>Serratia marcescens</i>	1
		<i>Sphingomonas paucimobilis</i>	1
		<i>Xanthomonas campestris</i>	1
		<i>Xanthomonas maltophilia</i>	3

medium with constant shaking at the growth temperature recommended for each species in the culture collection catalogue of the Laboratorium voor Microbiologie, Gent (LMG). Stock cultures were kept in glycerol (50% v/v) at -20°C and in saline at 4°C .

As positive and negative controls for the expression of the lipoprotein we used *E. coli* JE5513 (pLP12) (lpp⁻) and JE5513 (pKT240) (Cornelis *et al.*, 1989).

DNA preparation. Unless specifically stated, all techniques involving DNA manipulations were according to Sambrook *et al.* (1989). Overnight cultures were centrifuged at 12000 r.p.m. for 3 min in a microfuge and the bacterial pellet resuspended in 450 μl lysis buffer [50 mM-Tris/HCl pH 8, 25% (w/v) sucrose, 0.5 mg lysozyme ml^{-1}] and kept on ice. After 30 min, 35 μl 0.5 M-EDTA and 45 μl 10% (w/v) SDS were added and the tube incubated at 65°C for 15 min. This was followed by a first incubation at 37°C for 30 min with 37.5 μl 1 mg boiled RNAase A ml^{-1} (Sambrook *et al.*, 1989) followed by a second incubation for 1 h after addition of 50 μl 1 mg proteinase K ml^{-1} . The DNA was finally extracted by successive treatments with phenol, phenol/chloroform and chloroform before precipitation with 3 M-sodium acetate and 2-propanol. The DNA was stored at -20°C in 10 mM-Tris/HCl, 1 mM-EDTA (TE) buffer. Dilutions (1:100–1:1000) were used for the PCR reactions. When colony lysates were used, a single colony was resuspended in 50 μl H_2O and incubated in a boiling water bath for 10 min; 10 μl of a 1:100 or a 1:1000 dilution were taken for the PCR reaction.

PCR reaction. The PCR reaction was completed in microfuge tubes on a New Brunswick TC-1 thermocycler. The PCR mix (100 μl final volume) contained 49 μl sterile distilled water, 10 μl 10 $\times$ PCR buffer (500 mM-KCl, 100 mM-Tris/HCl pH 8.4, 15 mM-MgCl₂), 10 μl deoxy-

nucleotide mix [dGTP, dTTP, dATP, dCTP (2 mM each)], 10 μl diluted template DNA, 10 μl of each primer (50 pmol) and 1 μl enzyme (*Taq* from Pharmacia or Boehringer Mannheim, *Tub* from Amersham). The reaction mixture was layered with 70 μl mineral oil (Sigma) to avoid evaporation. The two primers (PS1, corresponding to the beginning of the open reading frame and PS2 corresponding to the end of the open reading frame), synthesized by phosphoramidite chemistry on an ABI 391EP DNA synthesizer (Applied Biosystems) were a 27- and a 21-mer, respectively. They had the following sequences: 5' ATGAACAACGTTCTGAAATTCCTGCT3', for PS1; 5' CTTGCGGCTGGCTTTTCCAG3', for PS2. Before adding the DNA polymerase (2 U per 100 μl of reaction mixture), each sample was denatured at 94°C for 5 min. We repeated the following amplification programme for 25–30 cycles: denaturation at 94°C for 1 min, annealing at 60°C or 64°C for 1 min, elongation at 72°C for 2 min. Ten μl from the amplified fragment were loaded on a 1.5% (w/v) agarose gel for electrophoresis. All PCR experiments included a positive control with *P. aeruginosa* PAO1 DNA and a negative control without DNA.

Restriction digestions. The digestion reactions were performed with 10 μl crude PCR amplified fragment in a total volume of 20 μl in sterile microfuge tubes containing 2 μl 10 $\times$ concentrated manufacturer's restriction buffer. The digestion mixture was incubated for 2 h at 37°C , 2 units of each restriction enzyme (Boehringer-Mannheim or Pharmacia-LKB) were added to each tube. Visualization of the restriction fragments was done on 5% (w/v) polyacrylamide gels in TBE buffer according to Sambrook *et al.* (1989).

Purification of outer membranes. Outer membranes were prepared as previously described using the Sarkosyl differential solubilization

Fig. 1. PAGE of amplified *oprI* genes and their restriction digest products from: *Pseudomonas aeruginosa* PAO1 (lanes 2 to 5); *P. reptilovora* (lanes 6–9); and *P. mendocina* (lanes 10–13). PCR products were either undigested (lanes 2, 6 and 10), or cut with restriction endonucleases *Hae*III (lanes 3, 7 and 11), *Pvu*II (lanes 4, 8 and 12) or *Sph*I (lanes 5, 9 and 13). Molecular mass markers, estimated from λ cut with *Pst*I (lanes 1 and 14), are indicated in base pairs.

method (Filip *et al.*, 1973; Cornelis *et al.*, 1989). The protein concentration was determined using a commercial kit (Bio-Rad) based on the Bradford colorimetric technique (Bradford, 1976).

Production of monoclonal antibodies. Adult female BALB/c mice were immunized in their footpads with outer-membrane preparations of *P. aeruginosa* PAO1 (Holmdahl *et al.*, 1985). Nine days later the popliteal lymph nodes from 5 mice were collected, the lymphocytes extracted and fused with NSO myeloma cells. After 10 d, the hybridoma were tested for the production of anti-OprI antibodies using an ELISA test with PAO1 outer membranes as antigen. Positive clones were further tested in ELISA with outer membranes from *E. coli* cells expressing the recombinant *Pseudomonas* OprI and from cells containing the plasmid vector pKT240 only. Clones reacting specifically with the lipoprotein were further subcloned before injection into mice for the production of ascites fluid.

Immunological detection of the lipoprotein. The lipoprotein was detected by colony blots using the monoclonal antibodies (mAbs) 15E11 or 3A9 (IgG2a). Each strain from different bacterial species was inoculated onto LB plates using sterile toothpicks and grown overnight at 28 °C. A nitrocellulose filter (Hybond C, Amersham) was then laid on the colonies, lifted and the cells lysed by laying the filter, colony side up, on a paper pad saturated with lysis buffer (25 mM-Tris/HCl pH 8, 1 mM-EDTA, 1 mg lysozyme ml⁻¹). After 30 min, the filter was laid on another paper pad saturated with 25 mM-Tris/HCl pH 8, 1% (w/v)

sarkosyl. The membrane was then saturated with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h, incubated in a 1:500 dilution of the 15E11 or 3A9 ascites fluid in PBS for 1 h at room temperature, washed three times in 0.1% PBS-Tween, incubated for 1 h with a 1:1000 dilution of horseradish peroxidase-conjugated protein A (Amersham), washed three times with PBS-Tween before detection with hydrogen peroxide and 4-chloro-1-naphthol (Bio-Rad). Western blots were performed according to Towbin *et al.* (1979) after separation of the outer membrane proteins on 15% (w/v) SDS-polyacrylamide gels. Dot blots on nitrocellulose filters were prepared by placing 100 µl of 1:100 diluted outer membranes in PBS into each well of a Manifold apparatus (Schleicher and Schuell). Antibodies used were the same as in the colony blots.

Results

PCR amplification of the *oprI* gene from *P. aeruginosa*

Thirty five *P. aeruginosa* strains tested for the *in vitro* amplification of the OprI lipoprotein gene consistently showed a unique amplification fragment of the expected size (249 bp) (Table 1, Table 2, Fig. 1, lane 1). Identical results were obtained for amplifications performed either on colony lysates or on purified DNA.

Fig. 2. Dot blot of outer membranes from different *Pseudomonas* and other species with the MAb 15E11. A1 to C3 (from left to right and top to bottom): *P. aeruginosa* strains PAO1 (A1), MH1 (A2), MH2 (A3), MH3 (A4), MH4 (A5), MH6 (A6), MH7 (A7), MH8 (A8), MH9 (A9), MH10 (A10), PA3 (A11), PA6 (A12), PA10 (B1), PA11 (B2), PA12 (B3), PA1022 (B4), PA1601 (B5), PA2019 (B6), PS3711 (B7), PS3870 (B8), DW1 (B9), DW2 (B10), DW3 (B11), DW4 (B12), DW5 (C1), DW6 (C2) and 7NSK2 (C3). Wells C4 to E1: other rRNA group I *Pseudomonas*: *P. alcaligenes* LMG 1224^T (C4), *P. agarici* LMG 2112^T (C5), *P. agarici* LMG 2113 (C6), *P. asplenii* LMG 2137^T (C7), *P. aureofaciens* LMG 1245^T (C8), *P. cichorii* LMG 2162^T (C9), *P. chlororaphis* T9 (C10), *P. fluorescens* ATCC 17400 (C11), *P. fragi* LMG 2191^T (C12), *P. fuscovaginae* LMG 2158^T (D1), *P. marginalis* LMG 2214 (D2), *P. marginalis* LMG 2238 (D3), *P. mendocina* LMG 1223^T (D4), *P. oleovorans* LMG 2229^T (D5), *P. pseudoalcaligenes* LMG 1225^T (D6), *P. putida* (A) LMG 2257^T (D7), *P. putida* (B) LMG 1246 (D8), *P. stutzeri* LMG 2333^T (D9), *P. syringae* LMG 1247 (D10), *P. taetrolens* LMG 2336^T (D11), *P. tolaasii* LMG 2340 (D12) and *P. tolaasii* LMG 2342^T (E1). Wells E2 to E9, other pseudomonads or related organisms: (*P.*) *andropogonis* LMG 2129^T (E2), (*P.*) *pickettii* LMG 6871 (E3), (*P.*) *solanacearum* LMG 2296 (E4), (*P.*) *solanacearum* LMG 2297 (E5), (*P.*) *solanacearum* LMG 2299^T (E6), (*P.*) *solanacearum* LMG 2306 (E7), *Xanthomonas maltophilia* LMG 958^T (E8) and *Azotobacter vinelandii* LMG 3884 (E9). Well F1: positive control with membranes of *E. coli* (pLPI2), well F2: negative control with membranes of *E. coli* (pKT240). All other wells are empty.

PCR amplification of the *oprI* gene in other members of the genus *Pseudomonas* and Gram-negative bacteria

From the results in Table 2, it is obvious that the *oprI* gene is conserved and restricted to the *Pseudomonas* species (21 tested) belonging to rRNA group I (Palleroni, 1984). All misnamed *Pseudomonas* species [21 strains tested, including *Comamonas acidovorans*, previously named (*Pseudomonas*) *acidovorans* and *Sphingomonas paucimobilis*, previously named (*Pseudomonas*) *paucimobilis* and *Xanthomonas maltophilia*, previously named (*Pseudomonas*) *maltophilia*] showed no amplification product. The conditions of amplification such as the annealing temperature were found to play a role in the specificity and signal intensity of the PCR reaction in *P. aeruginosa* and some other fluorescent pseudomonads. Exactly the same results were obtained at 60 °C or at 64 °C for the *P. aeruginosa* strains. However, for the other species (e.g. *P. fluorescens*, *P. stutzeri* and *P. fragi*) the amplification was weaker at 64 °C than at 60 °C (results not shown). None of the other Gram-negative bacteria tested gave a positive reaction in the amplification reaction. Again, similar amplification results were

obtained whether purified DNA or colony lysates were used as templates for PCR.

Detection of the lipoprotein with monoclonal antibodies

All of the strains tested by PCR were also screened for the presence of the lipoproteins by using mAbs raised against the lipoprotein of *P. aeruginosa* PAO1. mAb 15E11 was selected for further work since it reacted equally well in colony, dot and Western blots. The results obtained in colony blots and repeated in dot blots (Fig. 2) confirmed the observations of the previous PCR experiments (Table 2) in that only rRNA group I *Pseudomonas* species produced lipoprotein that reacted with mAb 15E11. A Western blot with outer membrane proteins from different PCR-positive rRNA group I *Pseudomonas* spp. showed that they all contained a lipoprotein of molecular mass about 12 kDa (Fig. 3). The presence of bands of higher molecular mass may be explained by the fact that some of the lipoprotein is bound to peptidoglycan of variable length. *Pseudomonas* spp. that were not members of rRNA group I did not contain this lipoprotein (e.g. Fig. 3, lane 12) and did not

Fig. 3. Western blot of outer membrane proteins separated on 15% SDS-PAGE. Lanes: 1, *P. aeruginosa* PAO1; 2, *P. aeruginosa* MH9; 3, *P. mendocina* LMG 1223^T; 4, *P. stutzeri* LMG 2333^T; 5, *P. putida* (A) LMG 2257^T; 6, *P. putida* (B) LMG 1246; 7, *P. fluorescens* ATCC 17400; 8, *P. aureofaciens* LMG 1245^T; 9, *P. cichorii* LMG 2162^T; 10, *P. syringae* LMG 1247; 11, *P. agarici* LMG 2113; 12, (*P.*) *solanacearum* LMG 2306. Molecular masses of protein markers are indicated on the left.

contain a protein band corresponding to the PAO1 lipoprotein in Coomassie Blue-stained SDS-polyacrylamide gels (results not shown).

Restriction analysis of the *oprI* amplified fragment

The 249 bp open reading frame of the lipoprotein gene contained unique *Hae*III, *Pvu*II and *Sph*I sites at positions 49, 112 and 228, respectively, from the first codon. In order to determine whether these restriction sites were phylogenetically conserved, we digested all PCR fragments with the three enzymes. Fragments were visualized after electrophoresis on 5% polyacrylamide gels. The restriction sites were found to be conserved in all strains of *P. aeruginosa*, *P. mendocina*, *P. oleovorans* and *P. pseudoalcaligenes* tested (Fig. 1; Table 3). The amplified *oprI*-containing DNA fragments from other species showed a variety of restriction fragment length polymorphisms (RFLPs). In some fragments (*P. fragi*, *P. stutzeri*, some strains of *P. putida*), all three sites were found to be absent while in others one or two sites were

conserved. Some amplified fragments were restricted by a given enzyme but not at the same location as in *P. aeruginosa*, as seen for *Pvu*II which cut the amplified fragment of *P. reptilovora* at a different location (Fig. 1, lane 8). Several strains of *P. putida* and *P. fluorescens* were tested giving different digestion patterns, confirming the well known heterogeneity of these two species for which different biotypes have been described (Palleroni, 1984).

Discussion

The sequence of the *oprI* gene from *P. aeruginosa* indicated that it differed from the other lipoprotein genes already cloned and sequenced from *Enterobacteriaceae* (Cornelis *et al.*, 1989), and suggested that it would be a suitable target for the detection of *P. aeruginosa* either by PCR or by immunological techniques involving mAbs. Recently, Saint-Onge *et al.* (1992) showed that amplification of the *oprI* open reading frame by PCR was possible in only a number of *Pseudomonas* species

Table 3. Restriction patterns of the *oprI* gene in rRNA group I *Pseudomonas* spp.

<i>Pseudomonas</i> spp.	Number of strains tested	Restriction sites		
		<i>Hae</i> III	<i>Pvu</i> II	<i>Sph</i> I
<i>P. aeruginosa</i>	17	+	+	+
<i>P. mendocina</i>	1	+	+	+
<i>P. oleovorans</i>	1	+	+	+
<i>P. pseudoalcaligenes</i>	1	+	+	+
<i>P. alcaligenes</i>	1	+	+	—
<i>P. fuscovaginae</i>	1	+	+	—
<i>P. reptilovora</i>	1	+	(+)	—
<i>P. aureofaciens</i>	1	+	—	—
<i>P. chlororaphis</i>	1	+	—	—
<i>P. fluorescens</i>	1	+	—	—
<i>P. marginalis</i>	1	+	—	—
<i>P. fragi</i>	1	—	—	—
<i>P. putida</i> A	1	—	—	—
<i>P. stutzeri</i>	1	—	—	—
<i>P. asplenii</i>	1	—	+	+
<i>P. fluorescens</i>	5	—	+	+
<i>P. corrugata</i>	1	—	—	+
<i>P. syringae</i>	1	—	—	+
<i>P. fluorescens</i>	3	—	+	—
<i>P. putida</i> B	1	—	+	—
<i>P. agarici</i>	1	+	—	+
<i>P. tolaasii</i>	1	+	—	+

(+), The *Pvu*II site was at a different position.

belonging to rRNA group I (Palleroni, 1984). In general, our data support and extend the observations of Saint-Onge *et al.* (1992); we included representatives of thirteen other *Pseudomonas* species from this rRNA group as well as a wide variety of so-called *Pseudomonas* species from other rRNA groups. However, in contrast to their study, none of the 35 *P. aeruginosa* isolates tested here was negative in the PCR reaction. *Chryseomonas luteola* is a free-living nitrogen-fixing species recently classified in the *Pseudomonas* rRNA group I (Reinhold-Hurch *et al.*, 1993). However, PCR and Western blot results presented here and by Saint-Onge *et al.* (1992) suggested that *OprI* and its gene in this organism were atypical for the group, even though Southern blot results of Saint-Onge *et al.* (1992) indicated that *C. luteola* contained DNA sequences that hybridized with *oprI* from *P. aeruginosa*. Thus, although rRNA group I *Pseudomonas* species showed positive amplification of the *oprI* gene, some exceptions may be expected as organisms from genera such as *Chryseomonas*, *Azotobacter* and *Azomonas* are classified close to rRNA group I pseudomonads (De Vos & De Ley, 1983).

It should be noted that sensitivity of the PCR technique described here means that great care has to be taken to avoid contamination which may produce false positive results, especially in general and clinical microbiological laboratories. In this study positive and

negative controls were included in each batch of PCR reactions, and results from PCR assays using both colony lysates and extracted DNAs as templates were confirmed with Western blots using mAb 15E11. Both techniques also showed negative reactions for the misnamed pseudomonads belonging to the '*Pseudomonas*' rRNA groups II, III and IV (Palleroni, 1984) and other Gram-negative species (Table 2). These findings are consistent with the known large phylogenetic distances between these rRNA groups and the authentic pseudomonads (De Vos & De Ley, 1983). Similar results were obtained by Siehn *et al.* (1990) who investigated the relatedness of *oprP* genes (the phosphate-specific porin in *P. aeruginosa*), and reported cross-hybridization only between members of rRNA group I and not with species belonging to the other groups.

Since amplification of the *oprI* gene by itself would not discriminate between rRNA group I *Pseudomonas* species, RFLP analysis of the PCR products was carried out using restriction endonucleases *Hae*III, *Pvu*II and *Sph*I. Our results showed eight different restriction profiles within the rRNA group I pseudomonads (Table 3). In all 17 *P. aeruginosa* strains tested, the three different restriction sites were present at the same sequence-predicted location. This is in excellent agreement with previous sequence data from three strains of *P. aeruginosa*, which showed very little variation in the *oprI* open reading frames (Cornelis *et al.*, 1989; Duchêne *et al.*, 1989; Saint-Onge *et al.*, 1992). Ullstrom *et al.* (1991) also found conservation of restriction sites in the *oprF* gene in the 17 serotypes of *P. aeruginosa*, with the exception of serotype 12. The same restriction pattern (Table 3) was found for *P. pseudoalcaligenes*, *P. oleovorans* and *P. mendocina*. DNA:DNA hybridization data (Palleroni *et al.*, 1972) showed that *P. aeruginosa* was most closely related to *P. pseudoalcaligenes*, which is in good agreement with our results. However, the same study showed only slight homology between *P. mendocina* and *P. aeruginosa*, and no DNA relatedness data were reported for *P. oleovorans*.

Although there was considerable restriction fragment length polymorphism in the amplified DNA fragments containing *oprI* at the species and sub-species level (Table 3), mAb 15E11 reacted with the *OprI* lipoprotein from all PCR-positive strains. This suggested that the epitope is conserved and that the nucleotide sequence variation may be confined to the third base of the codons. Finke *et al.* (1991) have already identified two epitopes in the *P. aeruginosa* lipoprotein, one at the N-terminal and one at the C-terminal.

In terms of the development of diagnostic methods and looking for markers of interest to the taxonomist we can conclude that the data on *oprI* presented here allowed differentiation of *P. aeruginosa* from all but

three (*P. mendocina*, *P. pseudoalcaligenes* and *P. oleovorans*) rRNA group I *Pseudomonas* species. However, a more detailed analysis would be required to determine if the OprI lipoprotein is suitable for the differentiation between the fluorescent pseudomonads at species or subspecies levels.

Our thanks to Professor R. Hamers for stimulating discussions. P.D.V. is indebted to the National Fund for Scientific Research (Belgium) for a position as Research Associate. We wish also to thank W. Verheulpen and J. Doumen for their assistance in computer work.

References

- BODEY, G. P., BOLIVAR, R., FAINSTEIN, V. & JADEJA, L. (1983). Infections caused by *Pseudomonas aeruginosa*. *Reviews of Infectious Diseases* **5**, 279–313.
- BODEY, G. P., JADEJA, L. & ELTING, L. (1985). *Pseudomonas* bacteraemia: retrospective analysis of 410 episodes. *Archives of Internal Medicine* **145**, 1621–1629.
- BOTZENHART, K. & RÜDEN, H. (1987). Hospital infections caused by *Pseudomonas aeruginosa*. *Antibiotics and Chemotherapy* **39**, 1–15.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **76**, 248–254.
- CORNELIS, P., BOUIA, A., GUYONVARCH, A., KAMMERER, B., HANNAERT, V. & HUBERT, J. C. (1989). Cloning and analysis of the gene for the major outer membrane lipoprotein from *Pseudomonas aeruginosa*. *Molecular Microbiology* **3**, 421–428.
- DAVISON, J. (1988). Plant beneficial bacteria. *Bio/Technology* **6**, 282–286.
- DE VOS, P. & DE LEY, J. (1983). Intra- and intergeneric similarities of *Pseudomonas* and *Xanthomonas* ribosomal ribonucleic acid cistrons. *International Journal of Systematic Bacteriology* **33**, 487–509.
- DE VOS, P., GOOR, M., GILLIS, M. & DE LEY, J. (1985). Ribosomal ribonucleic acid cistron similarities of phytopathogenic *Pseudomonas* species. *International Journal of Systematic Bacteriology* **35**, 169–184.
- DE VOS, P., VAN LANDSCHOOT, A., SEGERS, P., TYTGAT, R., GILLIS, M., BAUWENS, M., ROSSAU, R., GOOR, M., POT, B., KERSTERS, K., LIZZARAGA, P. & DE LEY, J. (1989). Genotypic relationships and taxonomic localization of unclassified *Pseudomonas* and *Pseudomonas*-like strains by deoxyribonucleic acid:ribosomal ribonucleic acid hybridizations. *International Journal of Systematic Bacteriology* **39**, 35–49.
- DUCHÊNE, M., BARRON, C., SCHWEIZER, A., VON SPECHT, B. U. & DOMDEY, H. (1989). *Pseudomonas aeruginosa* outer membrane lipoprotein I gene: molecular cloning, sequence, and expression in *Escherichia coli*. *Journal of Bacteriology* **171**, 4130–4137.
- FILIP, C., FLETCHER, G., WULFF, J. L. & EARHART, C. F. (1973). Solubilisation of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl-sarcosinate. *Journal of Bacteriology* **115**, 717–722.
- FINKE, M., MUTH, G., REICHEL, T., THOMA, M., DUCHÊNE, M., HUNGERER, K. D., DOMDEY, H. & VON SPECHT, B. U. (1991). Protection of immunosuppressed mice against infection with *Pseudomonas aeruginosa* by recombinant *P. aeruginosa* lipoprotein I and lipoprotein I-specific monoclonal antibodies. *Infection and Immunity* **59**, 1251–1254.
- GOVAN, J. R. W. (1986). *Pseudomonas aeruginosa* and cystic fibrosis: unusual bacterial adaptation and pathogenesis. *Microbiological Science* **3**, 302–308.
- HOIBY, N., DÖRING, G. & SCHIOTZ, P. O. (1987). Pathogenic mechanisms of chronic *Pseudomonas aeruginosa* infections in cystic fibrosis patients. *Antibiotics and Chemotherapy* **39**, 60–76.
- HOLMDAHL, R., MORAN, T. & ANDERSSON, M. (1985). A rapid and efficient immunization protocol for production of monoclonal antibodies reactive with autoantigens. *Journal of Immunological Methods* **83**, 379–384.
- MONDINO, B. J., WEISSMAN, B. A., FARB, M. D. & PETTIT, T. H. (1986). Corneal ulcers associated with daily wear and extended wear contact lenses. *American Journal of Ophthalmology* **102**, 58–65.
- PALLERONI, N. J. (1984). Genus I. *Pseudomonas* Migula 1894. In *Bergey's Manual of Systematic Bacteriology* vol. 1, pp. 141–199. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- PALLERONI, N. J., BALLARD, R. W., RALSTON, E. & DOUDOROFF, M. (1972). Deoxyribonucleic acid homologies among some *Pseudomonas* species. *Journal of Bacteriology* **110**, 1–11.
- REINHOLD-HUREK, B., HUREK, T., GILLIS, M., HOSTE, B., VANCANNEYT, M., KERSTERS, K. & DE LEY, J. (1993). *Azoarcus* gen. nov., nitrogen-fixing Proteobacteria associated with roots of Kallar Grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigenus* sp. nov. and *Azoarcus communis* sp. nov. *International Journal of Systematic Bacteriology* **43**, 574–584.
- SAINT-ONGE, A., ROMÉYER, F., LEBEL, P., MASSON, L. & BROUSSEAU, R. (1992). Specificity of the *Pseudomonas* PAO1 lipoprotein I gene as a DNA probe and PCR target region within the *Pseudomonadaceae*. *Journal of General Microbiology* **138**, 733–741.
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- SIEHNEL, R., MARTIN, N. L. & HANCOCK, R. E. W. (1990). Sequence and relatedness in other bacteria of the *Pseudomonas aeruginosa* OprP gene coding for the phosphate-specific porin P. *Molecular Microbiology* **4**, 831–838.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- ULLSTROM, C. A., SIEHNEL, R., WOODRUFF, W., STEINBACH, S. & HANCOCK, R. E. (1991). Conservation of the gene for outer membrane protein OprF in the family *Pseudomonadaceae*: sequence of the *Pseudomonas syringae* OprF gene. *Journal of Bacteriology* **173**, 768–775.
- WELLER, D. M. (1988). Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology* **26**, 379–407.