Pseudomonas pertucinogena sp. nov., an Organism Previously Misidentified as *Bordetella pertussis*

YOKO KAWAI AND EIKO YABUUCHI

Department of Bacteriology I, National Institute of Health, Shinagawa-ku, Tokyo, 141, Japan, and
Department of Microbiology, Kansai Medical School, Moriguchi-city, Osaka, 570, Japan

The morphological and biochemical attributes of two organisms, American Type Culture Collection (ATCC) strains 190 and 6627, originally identified as *Bordetella pertussis*, were studied. The guanine-plus-cytosine content of the deoxyribonucleic acid of each strain is 60 mol%. The deoxyribonucleic acid homology between the two strains is 99%. The strains are quite similar phenotypically and genetically, and they are regarded here as belonging to one and the same species. Although the two strains produce pertucin, a bacteriocin active against *B. pertussis* strains in the smooth phase, they possess the characteristics of the genus *Pseudomonas* as defined by Shewan and Haynes. However, ATCC 190 and 6627 could not be placed in any of the *Pseudomonas* species described in the literature. Therefore, these strains are regarded as belonging to a new species, for which we propose the new name *Pseudomonas pertucinogena*. The type strain of *P. pertucinogena* is ATCC 190.

For many years, American Type Culture Collection (ATCC) 190 and ATCC 6627, two motile, polar monotrichous organisms, have been unwittingly accepted as rough phase IV *Bordetella pertussis* strains (21); Kawai (18) and Aprile (2) recognized this enigma. The two strains produce pertucin, a bacteriocin that inhibits the growth of smooth strains of *B. pertussis*. Although the colonial morphology of these strains is similar to that of rough strains of *B. pertussis*, the motility, flagellar morphology, and guanine-plus-cytosine (G+C) content of their deoxyribonucleic acid (DNA) distinguish them from smooth and rough strains of *B. pertussis*. Their polar monotrichous anatomy and other attributes suggest that they are pseudomonads. In our opinion, these strains do not belong to any of the *Pseudomonas* species described in *Bergey's Manual of Determinative Bacteriology* (6). Although these strains were referred to by the name *Pseudomonas pertucinogena* in a previous publication (19), the name was only mentioned incidentally and therefore was not validly published. The purpose of this paper is to effect the valid publication of the name and to provide a description of the species.

MATERIALS AND METHODS

Bacterial strains. The designations and sources of the 16 strains included in this study are listed in Table 1.

Methods. The morphological, physiological, and biochemical characteristics of ATCC 190 and ATCC 6627 were determined primarily by methods described previously (40). Acid production from glucose and 25 additional carbon compounds was deter-

mined in oxidation-fermentation basal medium (Difco 688) and cystine-tryptone agar (Eiken). Antibiotic susceptibility tests were performed as described previously (40). In determinations of sole carbon and energy sources, each of 47 carbon compounds was added to a standard mineral base (34) in a concentration of 0.1%. Growth was harvested from a 24-h-old brain heart infusion agar culture and suspended in sterile saline solution to a concentration of 10^7 cells per ml. Tubes of the 47 liquid media were inoculated with 0.05 ml of this suspension, incubated at 37 C, and observed daily for 5 days. Culture media that became turbid within 24 to 48 h were serially subcultured.

G+C content of DNA. DNA extracted from cells grown on Cohen and Wheeler medium (8) slants was purified (36). The ultraviolet (UV) absorption spectrum of the DNA was determined in a recording spectrophotometer (Hitachi model 124). Phosphorus in the DNA was analyzed by Nakamura's modification (23) of the Allen method (1). Nitrogen was determined by the micro-Kjeldahl method. The purity of the DNA was confirmed by UV spectroscopy and by the N/P ratio. The melting temperature (T_m) of the DNA was determined in 0.015 M sodium chloride-0.0015 M sodium citrate solution ($0.1 \times$ SSC) with a recording spectrophotometer (Shimazu model MPS-50L), by the method of Marmur and Doty (22) and Silvestri and Hill (33). The DNA was hydrolyzed with 88% formic acid at 175 C for 30 min (39), and the paper chromatogram was developed with the solvent system isopropanol-6 N hydrochloric acid-water (68:16.4:15.6, vol/vol/vol). UV spectroscopy was performed as described by Ulitzur (37).

DNA homology. All of the tested strains (see Table 7) except *Pseudomonas fluorescens* ATCC 13525 and *Pseudomonas putida* ATCC 12633 were grown at 37 C with shaking to the logarithmic phase of growth. The growth medium had the following composition: 2% Casamino Acids solution treated

TABLE 1. List of strains included in this study^a

Strain received as	Received from	Corresponding strain designation			Status
		ATCC	KM	Other	
<i>Bordetella pertussis</i> , phase IV	ATCC	190	1319		
<i>B. pertussis</i> , phase IV	ATCC	6627	1320		
<i>B. pertussis</i> , phase I	Liu	8467			
<i>B. pertussis</i> , phase I	NIHJ			18323	
<i>B. pertussis</i> , phase III	IK			Sakurayashiki	
<i>B. pertussis</i> , phase III	IK			3 B	
<i>B. pertussis</i> , phase IV	IK			Sakurayashiki	
<i>B. pertussis</i> , phase IV	IK			Kudo	
<i>B. parapertussis</i>	NIHJ			17903	
<i>B. bronchicanis</i>	NIHJ			214	
<i>Pseudomonas aeruginosa</i>	ATCC	10145	274		Neotype
<i>P. fluorescens</i>	ATCC	13525	361		Neotype
<i>P. alcaligenes</i>	ATCC	14909	362		Neotype
<i>P. pseudocalcaligenes</i>	ATCC	17440	363		Type
<i>P. maltophilia</i>	ATCC	13637	418		Type
<i>P. putida</i>	ATCC	12633	888		Neotype

^a Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; IK, Institute of Kitasato, Tokyo; KM, Kansai Medical School Culture Collection, Osaka; NIHJ, National Institute of Health, Tokyo.

with charcoal, 500 ml; NaCl, 2.5 g; MgCl₂·6 H₂O, 0.04 g; FeSO₄·7 H₂O, 0.01 g; L-cystine, 0.025 g; soluble starch, 1.5 g; K₂HPO₄, 4 g; KH₂PO₄, 2 g; 10⁻³ M nicotinic acid solution, 10 ml; biotin (5 µg/ml), 0.1 ml; and distilled water to make the total volume 1,000 ml (pH 7.0). [8-¹⁴C]guanine or [8-³H]guanine was added at a level of 0.2 µCi/ml, respectively. The *P. fluorescens* and *P. putida* strains were grown at 30 C. The DNA was extracted and purified as described previously (31, 36). DNA-DNA reassociation was performed by the membrane filter technique with formamide at low temperature (24, 36). Sheared, denatured ¹⁴C-labeled DNA (SD-DNA) of strains ATCC 190 and ATCC 17440 and ³H-labeled and immobilized DNA (IM-DNA) from each of the 12 strains tested were reassociated in a vibrating incubator at 45 or 37 C for 24 h. Each homology was performed in duplicate. The relative reassociation value of the DNA was calculated from the amount of SD-DNA reassociated per 2-µg unit of IM-DNA retained on the membrane.

Reassociated homologous DNA of strain ATCC 6627 was compared with the reassociated heterologous DNA of ATCC 6627-ATCC 190 and ATCC 6627-ATCC 13637 for the thermal dissociation profile by the method of Toyama et al. (36).

Serology. Rabbit antisera, homologous titers 1:6,400 and 1:3,200, specific for *B. pertussis* strains in phases I and III, respectively, were used for testing for the tube agglutination of ATCC 190 and ATCC 6627.

Animal inoculation. Strains ATCC 190 and ATCC 6627 were inoculated intravenously and intraperitoneally into mice. Cultures of each strain were suspended in saline at four concentrations (10⁸, 10⁹, 10¹⁰, and 10¹¹ cells/ml) and were used as inocula either as intact cell suspensions or cells disrupted by sonic treatment at 10 kcycles. A 0.1-ml volume of each preparation was injected into the shaved skin

on the back of two guinea pigs, and 0.25 ml was injected intravenously or intraperitoneally into a mouse.

RESULTS

ATCC strains 190 and 6627 were gram-negative, non-encapsulated, asporogenous, straight rods. Soma size was about 0.4 × 1.1 µm. Cells of both strains from solid and liquid media were motile and polar monotrichous. The flagellar morphology of each strain is shown in Fig. 1.

Both strains produced a slight, homogeneous turbidity in brain heart infusion broth after 24 h at 30 C. The turbidity increased after 2 days of incubation, but the broth did not become as turbid as that of a 24-h-old culture of *P. aeruginosa*. Colonies on Bordet-Gengou medium after 24 h of incubation at 37 C were grayish, 1 mm in diameter, and mimicked the colonies of rough phase IV strains of *B. pertussis*. Colonies of the two strains were less than 1 mm in diameter, semitranslucent, entire, smooth, and glistening on brain heart infusion agar, Trypticase soy agar, heart infusion agar, and nutrient agar. The biochemical reactions of the two strains are summarized in Tables 2 and 3. Substrates utilized and those not utilized by the two strains as the sole carbon and energy sources are listed in Table 4.

Both strains were susceptible to 12 antibiotics, including penicillin, and were resistant to novobiocin (Table 5).

Specific antisera for *B. pertussis* failed to agglutinate either of the two strains.

Dermal necrosis in guinea pigs appeared at 24 h after injection of ultrasonically disrupted

FIG. 1. *Pseudomonas pertucinogena* ATCC 190 (a) and 6627 (b). Broth culture. Leifson flagella stain after formalin fixation. $\times 2,000$.

cell suspensions of ATCC 190 and ATCC 6627 at a concentration of 10^{11} cells/ml. Sonically disrupted suspensions in lower concentrations of cells and whole-cell suspensions in four concentrations did not produce dermal necrosis in the guinea pig. Whole or ultrasonically disrupted cells of both strains at concentrations of 10^{11} cells per ml injected either intraperitoneally or intravenously appeared to weaken mice within 24 h; the mice recovered and appeared healthy within 48 h. Whole or sonically disrupted cells in lower concentrations did not kill mice during 10 days of observation.

The G+C content of the DNA of ATCC 190 and of ATCC 6627 was 60 mol%, whereas the G+C content of the DNAs of eight strains of three *Bordetella* species was around 67 mol% (Table 6). DNA homology between strains ATCC 190 and ATCC 6627 was 99% at 45 C, the specific hybrid temperature. DNA homologies between ATCC 190 and the type or neotype strains of six *Pseudomonas* species at 45 C are listed in Table 7. The thermal elution profile of ATCC 6627 SD-DNA reassociated with ATCC 190 IM-DNA was identical to that of the SD-DNA of ATCC 6627 reassociated with homologous IM-DNA (Fig. 2).

DISCUSSION

During a survey for bacteriophages of *Bordetella pertussis*, Litkenhous and Liu (21) noted that strains ATCC 190 and ATCC 6627 produced a bacteriocin that inhibited the growth of *B. pertussis* strains. Kawai (19) reported the purification and characterization of pertucins produced by ATCC 190 and 6627.

The ATCC received "*B. pertussis*" strain 2205 from the Eli Lilly Co. during March 1925, and this strain was assigned the ATCC accession

TABLE 2. Characteristics of ATCC 190 and ATCC 6627^a

Characteristic	ATCC190	ATCC 6627
Gram-negative, rod-shaped cells	+	+
Single polar flagellum	+	+
Motility	+	+
Indophenol oxidase and catalase	+	+
Growth on LD agar and SS agar	—	—
Growth on pseudosel agar and on NAC agar	—	—
Growth in BHI at 41 C	+	+
Growth in BHI at pH 4.5	—	—
Growth in BHI containing 3% NaCl	+	+
Growth in KCN broth	—	—
Indole, methyl red, acetyl-methylcarbinol	—	—
Simmons citrate, Christensen citrate	—	—
Malonate	—	—
Kligler iron agar slant, acid	—	—
Kligler iron agar butt, acid and H ₂ S	—	—
H ₂ S, lead acetate paper	+	+
Nitrate reduction to nitrite	—	—
Pigment production, water soluble	—	—
Hemolysis around surface colonies and green discoloration of rabbit blood	—	—
Lysine decarboxylase, ninhydrin	—	—
L-Lysine, L-ornithine decarboxylase, Møller	—	—
L-Arginine dihydrolase, Møller	—	—
Phenylalanine deaminase	+	+
Acyamidase, urease, phosphatase	—	—
Deoxyribonuclease, extracellular	—	—
Hydrolysis of gelatin, starch, Tween 80	—	—
Growth in gluconate broth	—	—
2-Ketogluconate production	NT	NT
Growth at the expense of <i>p</i> -hydroxybenzoate	—	—
Cleavage of protocathecuat	NT	NT
Growth at the expense of β -hydroxybutyrate	+	+
Accumulation of poly- β -hydroxybutyrate	—	—

^a Symbols: +, positive reaction; —, negative reaction. Abbreviations: NT, not tested; BHI, brain heart infusion; SS, salmonella-shigella.

TABLE 3. Acid production by ATCC 190 and ATCC 6627 from carbon compounds^a

Substrates	ATCC 190		ATCC 6627	
	OF	CTA	OF	CTA
Ethanol, glycerol	—	—	—	—
Adonitol, dulcitol	—	—	—	—
Inositol, mannitol, sorbitol	—	—	—	—
Xylose	NC	WK ₁₀ ^b	NC	WK ₁₀
D-Arabinose	NC	+ ₂₀	NC	WK ₁₀
L-Arabinose	NC	NC	NC	WK ₂₀
D-Ribose	NC	WK ₂₀	WK ₂₀	WK ₂₀
Rhamnose	—	—	—	—
Glucose	WK ₇	+ ₄	WK ₈	+ ₄
Fructose	—	—	—	—
Galactose	NC	+ ₂₀	NC	WK ₂₀
Mannose	NC	WK ₂₀	NC	NC
Lactose, maltose	—	—	—	—
Cellobiose, melibiose sucrose, trehalose	—	—	—	—
Raffinose, melezitose	—	—	—	—
Inulin, salicin	—	—	—	—
Basal medium, control alkaline	+	+	+	+

^a Symbols: +, positive reaction; —, negative reaction. Abbreviations: NC, no change, WK, weakly positive reaction; CTA, cystine-tryptone agar (Eiken); OF, oxidation-fermentation basal medium (Difco 688).

^b Figures indicate days required to obtain positive or weakly positive reaction.

number 190. On 26 October 1953, ATCC 190 was replaced by a strain, in the rough phase, received from Pearl R. Kendrick (University of Michigan School of Public Health). "*B. pertussis*," phase IV, was deposited by R. S. Muckenfuss, New York Department of Health, in the ATCC as strain 6627 on 3 May 1938.

Although the sources of isolation of the two strains were not recorded, their identification as strains of *B. pertussis* suggests that they were isolated from the human respiratory tract. Although identified as strains of *B. pertussis*, ATCC 190 and ATCC 6627 are motile by means of a single polar flagellum. They require molecular oxygen to oxidize glucose and other carbohydrates to acid without gas production, they fail to obtain energy by fermentation or photosynthetic metabolism, and the G+C content of their DNA is about 60 mol%. Because of these findings, it seems reasonable to conclude that

ATCC 190 and ATCC 6627 are pseudomonads, not bordetellas. The morphological, physiological, and biochemical characters, the DNA homologies (99%), and the thermal dissociation profile of reassociated DNA indicated that the two strains belong to one and the same species.

TABLE 4. Utilization of substrates by ATCC 190 and ATCC 6627 as the sole sources of carbon and energy

Utilized		
Amino acid		
L- α -Alanine		
Organic acid		
Pyruvate		
Succinate		
Oxaloacetate		
β -Hydroxybutyrate		
Not utilized		
Amino acid	Carbohydrate	Alcohol
L-Glycine	D-Arabinose	Ethanol
L-Leucine	L-Arabinose	Polyalcohol
L-Isoleucine	D-Ribose	Adonitol
L-Serine	Xylose	Inositol
L-Threonine	Rhamnose	Mannitol
L-Valine	Glucose	Sorbitol
L-Aspartic acid	Fructose	Dulcitol
L-Glutamic acid	Galactose	Glycerol
L-Proline	Mannose	
L-Tryptophane	Lactose	
L-Phenylalanine	Maltose	
L-Tyrosine	Cellobiose	
L-Histidine	Sucrose	
L-Arginine	Trehalose	
L-Lysine	Raffinose	
L-Ornithine	Salicin	
L-Methionine	Inulin	
α -Amino-n-butyric acid		
β -Amino-n-butyric acid		
γ -Amino-n-butyric acid		

TABLE 5. Susceptibilities of ATCC 190 and ATCC 6627 to antibiotics^a

Antibiotic	ATCC 190	ATCC 6627
Ampicillin	+++	+++
Chloramphenicol	+++	+++
Colistin	+++	+++
Erythromycin	++	+++
Gentamicin	+++	+++
Kanamycin	+++	+++
Nalidixic acid	++	++
Novobiocin	—	—
Penicillin	++	++
Polymyxin B	+++	+++
Streptomycin	+++	+++
Tetracycline	+++	++

^a The degree of susceptibility is expressed as +++, ++, or +, in decreasing order; —, resistant. The amount of antibiotic used and the criteria for determining susceptibility have been previously described (40).

TABLE 6. G+C contents of the DNAs of the strains studied

DNA prepared from:	G+C content (mol%)		
	Melting temperature method	Hydrolysis and chromatography method	UV spectroscopy
" <i>Bordetella pertussis</i> " ATCC 190	60	59.5	59.5
" <i>Bordetella pertussis</i> " ATCC 6627	59	60	61
<i>Bordetella pertussis</i> ATCC 8467, phase I	68	66	68
<i>B. pertussis</i> 18323, phase I	68	67	67
<i>B. pertussis</i> Sakurayashiki, phase III	67	66	67
<i>B. pertussis</i> 3 B, phase III	70		
<i>B. pertussis</i> Sakurayashiki, phase IV	66	67	67
<i>B. pertussis</i> Kudo, phase IV	70		
<i>Bordetella parapertussis</i> 17903		66	66
<i>Bordetella bronchicanis</i> 214		66	67

TABLE 7. DNA homologies of 11 strains with "*Bordetella pertussis*" ATCC 190 and *Pseudomonas pseudoalcaligenes* ATCC 17440

Strains tested	Homology index (%)		
	ATCC 190		ATCC 17440 (45 C)
	45 C	37 C	
" <i>Bordetella pertussis</i> " ATCC 190	100	100	27
" <i>Bordetella pertussis</i> " ATCC 6627	99	92	26
<i>Pseudomonas pseudoalcaligenes</i> ATCC 17440	17	31	100
<i>P. aeruginosa</i> ATCC 10145	15	22	26
<i>P. fluorescens</i> ATCC 13525	11	20	24
<i>P. alcaligenes</i> ATCC 14909	13	22	28
<i>P. maltophilia</i> ATCC 13637	8	18	6
<i>P. putida</i> ATCC 12633	11	28	19
<i>Bordetella pertussis</i> 18323, phase I	9	15	14
<i>B. pertussis</i> ATCC 8467, phase I	7	12	8
<i>B. pertussis</i> Sakurayashiki, phase III	9	20	13
<i>B. pertussis</i> Sakurayashiki, phase IV	8	32	16

Kawai (18) described the single polar flagellation of ATCC 190 and ATCC 6627 and proposed that they be placed in the genus *Pseudomonas*. Aprile (2) suggested that ATCC 190 belongs to *Lophomonas* (10) because it possesses one or two polar flagella and fails to oxidize glucose. However, *Lophomonas* is an illegitimate bacterial generic name (7), and Davis and Park (9)

FIG. 2. Thermal dissociation profile of reassociated DNA. Symbols: ●, homologous DNA (ATCC 6627); ×, hybridized DNA between ATCC 6627 and ATCC 190; Δ, hybridized DNA between ATCC 6627 and ATCC 13637. A membrane-bearing reassociated DNA was heated in $0.1 \times$ SSC for 10 min at various temperatures from 60 to 95°C (5°C increments). Dissociated DNA, after addition of carrier DNA, was precipitated with 5% trichloroacetic acid, collected on a membrane filter, and counted (14 C radioactivity).

proposed a new generic name, *Comamonas*, to replace it. *Comamonas terrigena* (Günther) Hugh (12) is the only species recognized in the genus *Comamonas*. In contrast to the polar monotrichous morphology of ATCC 190 and ATCC 6627, the neotype strain for *C. terrigena* has a tuft of polar flagella and produces a basic reaction in oxidation-fermentation basal medium containing glucose or any of 25 other carbon compounds which were tested for ATCC 190 and ATCC 6627.

Since ATCC 190 and ATCC 6627 are of clinical origin, they were compared with the type or neotype strains of six *Pseudomonas* species frequently encountered in clinical specimens (14–17). DNA-DNA hybridization experiments showed a high degree of homology between ATCC 190 and ATCC 6627 and a low degree of homology between the two strains and four strains of *B. pertussis* or the six *Pseudomonas* strains (Table 7). Furthermore, the two strains could not be identified as belonging to any of the currently recognized or recently described *Pseudomonas* species (3, 4, 6, 11, 13, 20, 25–30, 34, 35, 38, 40).

Thus, strains ATCC 190 and ATCC 6627 appear to belong to a new species, for which we propose the name *Pseudomonas pertucinogena* (M. L. noun *pertucin* pertucin, a bacteriocin that inhibits smooth strains of *Bordetella pertussis*; Gr.v. *gennao* to produce; M. L. adj. *pertucinogenus* producing pertucin). ATCC 190 is designated as the type strain for the species.

In closing, it should be noted that although Bordet-Gengou medium was specifically designated for the isolation of *B. pertussis* strains from human respiratory tract specimens, it does not inhibit the growth of all other bacteria in these specimens. Even if the medium contains an appropriate quantity of penicillin to inhibit the growth of gram-positive cocci, it is not a perfect selective isolation medium for *B. pertussis* strains. *Bordetella parapertussis*, *Bordetella bronchicantis*, and many other aerobic gram-negative rods grow on Bordet-Gengou medium containing penicillin. Colonies on this medium which resemble those of *B. pertussis* should be transferred to a peptone broth, such as brain heart infusion, without blood or charcoal, and should be identified on the basis of morphological, physiological, and biochemical properties. The slide agglutination test, performed with specific antipertussis serum, is a confirmatory tool for the identification of *B. pertussis* strains.

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REPRINT REQUESTS

Address reprint requests to: Yoko Kawai, Department of Bacteriology I, National Institute of Health, 10-35, 2-chome, Kamiosaki, Shinagawa-ku, Tokyo, 141, Japan.

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