Reclassification of *Paenibacillus* (formerly *Bacillus*) *pulvificiens* (Nakamura 1984) Ash et al. 1994, a Later Subjective Synonym of *Paenibacillus* (formerly *Bacillus*) *larvae* (White 1906) Ash et al. 1994, as a Subspecies of *P. larvae*, with Emended Descriptions of *P. larvae* as *P. larvae* subsp. *larvae* and *P. larvae* subsp. *pulvificiens*

M. HEYNDRIX,¹ K. VANDEMEULEBROECKE,¹ B. HOSTE,¹ P. JANSSEN,¹ K. KERSTERS,¹
P. DE VOS,^{1*} N. A. LOGAN,² N. ALI,³ AND R. C. W. BERKELEY³

Laboratorium voor Microbiologie, Universiteit Gent, B-9000 Ghent, Belgium,¹ and Department of Biological Sciences, Glasgow Caledonian University, Glasgow G4 0BA,² and Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD,³ United Kingdom

A polyphasic taxonomic study of four strains of *Paenibacillus larvae* and four strains of *Paenibacillus pulvificiens* (including duplicates of both type strains) supported the reclassification of both former *Bacillus* species into one species, *P. larvae*. Our conclusions were based on morphological and Analytab Products (API) tests, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins, gas chromatography of methylated fatty acids, pyrolysis mass spectrometry, DNA-DNA binding, and the following genomic fingerprinting methods: amplified ribosomal DNA restriction analysis, random amplified polymorphic DNA analysis, and AFLP analysis. The last method is a novel high-resolution DNA fingerprinting technique based on the selective amplification of restriction fragments. Despite more than 90% DNA relatedness between the strains studied, SDS-PAGE of whole-cell proteins, biochemical tests, and DNA fingerprinting (AFLP) distinguished between the *P. larvae* and *P. pulvificiens* strains at the subspecies level. Taking this evidence along with differences in pathogenicity, we propose to reclassify the honeybee pathogens *P. larvae* and *P. pulvificiens* as *P. larvae* subsp. *larvae* and *P. larvae* subsp. *pulvificiens*. An emended description of the species and descriptions of the subspecies are given. The type strains are *P. larvae* subsp. *larvae* ATCC 9545 (LMG 9820) and *P. larvae* subsp. *pulvificiens* NRRL B-3685 (LMG 6911 and ATCC 13537).

In 1906, White (29) described the species *Bacillus larvae*, an obligate pathogen of honeybee (*Apis mellifera*) larvae causing American foulbrood disease. As these organisms required special media for cultivation in vitro (media which underwent progressive refinement from a macerated, healthy bee larva medium to a tryptone-glucose-yeast extract medium), their inability to grow on ordinary media such as nutrient agar was considered to be a valuable diagnostic characteristic (9).

In 1950, Katznelson (16) described a further species, *Bacillus pulvificiens*, strains of which had been isolated from honeybee larvae in association with a rare disease called powdery scale. This condition is characterized by a powdery decay of the larvae, which are removed from the hive prior to sporulation of the pathogen; in the more common American foulbrood disease, the glue-like consistency of the infected larvae results in the persistence of the infectious scale within the cells. Although it resembled *B. larvae*, *B. pulvificiens* could be distinguished by its abilities to grow in ordinary nutrient media after serial transfers, to grow at 20°C, and to produce an orange pigment, as well as by its different pathogenicity.

Subsequently, other authors reported spore surface contour and serological differences between *B. larvae* and *B. pulvificiens* (9), but Gordon and colleagues (9) felt unable to recognize *B. pulvificiens* taxonomically because the seven strains

available to them for study were all isolated from the same source. This shortage of strains led to the neglect of *B. pulvificiens* in later numerical taxonomic studies and therefore to its omission from the "Approved Lists of Bacterial Names" (25) and loss of its standing in bacteriological nomenclature.

Following a DNA relatedness study of 24 strains received as "*B. pulvificiens*," Nakamura (21) revived the name *B. pulvificiens* for the larger of two groups which showed a low level of homology with the type strains of *Bacillus alvei*, *B. larvae*, and *Bacillus laterosporus*.

In 1992, the Subcommittee on the Taxonomy of the Genus *Bacillus* of the International Committee on Systematic Bacteriology decided to undertake a collaborative polyphasic taxonomic study of the genus *Bacillus*, with the establishment of minimal standards for the description of species and revision of the nomenclature among its objectives (14). Our research work revealed a close relationship between strains of *B. larvae* and *B. pulvificiens* (species which have been transferred to the new genus *Paenibacillus* [2]), and these organisms were therefore studied more closely in order to determine the exact taxonomic and nomenclatural implications.

MATERIALS AND METHODS

Strains and media. The designations of the strains, their origins and taxonomic status, and the different methods applied are compiled in Table 1. The authenticity of the type strains of *Paenibacillus larvae* and *Paenibacillus pulvificiens* was verified by studying duplicate cultures obtained from the American Type Culture Collection. Strains were grown at 30°C for 24 to 48 h on nutrient agar (pH 7.4) containing 0.1% (wt/vol) Lab-Lemco beef extract (Oxoid), 0.2% (wt/vol) yeast extract (Oxoid), 0.5% (wt/vol) peptone (Oxoid), and 0.5% (wt/vol) NaCl, with

* Corresponding author. Mailing address: Vakgroep BFM WE10V, Laboratorium voor Microbiologie, Universiteit Gent, K. L. Ledeganckstraat, 35, B-9000 Ghent, Belgium. Phone: 32 (0) 9 264 5110. Fax: 32 (0) 9 264 5346. Electronic mail address: Paul.Devos@rug.ac.be.

TABLE 1. *P. pulvifaciens* and *P. larvae* strains and strains of other *Paenibacillus* species used and overview of methods applied to characterize them

Strain ^a	Other designation(s) ^a	Source ^a	Methods applied ^b							
			ARDRA	API	PAGE	FAME	AFLP	DNA homology	Py-MS	RAPD
<i>P. larvae</i>										
LMG 9820 ^T	ATCC 9545 ^T	ATCC ^c	+	+	+	+	+	+	+	+
LMG 15969 ^T	ATCC 9545 ^T	ATCC ^c		+	+		+		+	+
LMG 14425	ATCC 25747	ATCC	+	+	+	+	+	+	+	+
LMG 14426	ATCC 25748	ATCC	+	+	+	+	+	+	+	+
<i>P. pulvifaciens</i>										
LMG 6911 ^T	NCIMB 11201 ^T , NRRL B-3685 ^T	NCIMB	+	+	+	+	+	+	+	+
LMG 15974 ^T	ATCC 13537 ^T	ATCC		+	+		+		+	+
LMG 14427	ATCC 25367	ATCC	+	+	+	+	+	+	+	+
LMG 14428	ATCC 25368	ATCC	+	+	+	+	+	+	+	+
<i>P. alvei</i>										
LMG 13253 ^T	B0016 ^T , DSM 29 ^T , ATCC 6344 ^T	DSM	+	+	+				+	+
LMG 13258	B0810, NCIMB 8199, NRRL B-384	NCIMB	+	+	+				+	
<i>P. validus</i>										
LMG 11161 ^T	B1397 ^T , DSM 3037 ^T	DSM	+	+	+				+	+
LMG 14019	B1393, NRRL NRS-839A	Nakamura	+	+	+		+	+		
LMG 14020	B1394, NRRL NRS-625	Nakamura			+					
LMG 14470	Pichinoty Tri 4, DSM 6545	DSM			+		+			
LMG 14666	B1403, Pichinoty Pohb 1	Pichinoty						+		
LMG 14668	B1405, Pichinoty Tri 5	Pichinoty			+					
LMG 9817	B1395, Pichinoty Q1, ATCC 29948	ATCC	+	+	+		+		+	+
<i>P. polymyxa</i>										
LMG 13294 ^T	B0011 ^T , DSM 36 ^T , ATCC 842 ^T	DSM	+	+	+				+	+
LMG 13296	B0264, T. Gibson 43	Gibson	+	+	+				+	+
<i>P. pabuli</i>										
LMG 14017	B1391, NRRL BD-536	Nakamura	+	+			+			
LMG 14671	B1390, NRRL B-1488	Nakamura	+	+			+			

^a ATCC, American Type Culture Collection, Rockville, Md.; B, N. A. Logan *Bacillus* collection, Glasgow Caledonian University, Glasgow, United Kingdom; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; T. Gibson, T. Gibson collection, held at University of Bristol, Bristol, United Kingdom; LMG, Culture Collection Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; Nakamura, L. K. Nakamura, U.S. Department of Agriculture, Peoria, Ill.; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen University, Aberdeen, United Kingdom; NRRL, Northern Regional Research Laboratory collection, Peoria, Ill.; NRS, N. R. Smith collection; Pichinoty, F. Pichinoty, UER Scientifique de Luminy, Marseille, France.

^b API System tests and other biochemical and morphological observations.

^c Two independent subcultures of *P. larvae* ATCC 9545^T arrived in the LMG collection, in 1990 (LMG 9820^T) and 1995 (LMG 15969^T).

the exception of *P. larvae* strains, which were cultivated at 37°C for 24 to 48 h either on the same medium supplemented with 1 mg of thiamine hydrochloride per liter or on Columbia blood agar (pH 7.1 to 7.5) containing 2.3% (wt/vol) special peptone (Oxoid L72), 0.5% (wt/vol) NaCl, 0.5% (wt/vol) glucose, 0.1% (wt/vol) starch, 0.03% (wt/vol) cysteine hydrochloride, 1% (wt/vol) agar no. 1 (Oxoid L11), and 5% (vol/vol) sterile horse blood (Sanofi Diagnostics Pasteur). The strains were checked for purity by plating and microscopic examination and were maintained as lyophilized cultures and at 4°C on slopes of nutrient agar containing 5 mg of MnSO₄ · 4H₂O per liter to enhance sporulation; this medium was supplemented with 1 mg of thiamine hydrochloride per liter for *P. larvae*.

SDS-PAGE of whole-cell proteins. Material for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was obtained by growing cells of *P. larvae* and *P. pulvifaciens* in petri dishes on Columbia blood agar at 30 and 37°C for 24 h and on nutrient agar supplemented with 1% (wt/vol) glucose (pH 7.4) at 30 and 37°C for 24 h after a preculture on Columbia blood agar. Cells of *Paenibacillus alvei*, *Paenibacillus polymyxa*, and *Paenibacillus validus* were grown on nutrient agar with 1% (wt/vol) glucose (pH 7.4) at 30°C for 24 h. The methods used for protein extraction, electrophoresis, and cluster analysis of the protein profiles have been described previously (12, 24, 27).

Gas chromatographic analysis of methylated fatty acids. Cells were grown on Trypticase soy agar (TSA) at 28°C for 24 h as recommended by the Microbial Identification System (MIS) (Microbial ID, Inc., Newark, Del.). For growth of *P. larvae*, this procedure was modified as follows: 1 mg of thiamine hydrochloride per liter was added to TSA and incubation was done at 37°C for 24 h. Fatty acid methyl ester (FAME) analysis was also performed for all strains grown on Columbia blood agar at 28 and 37°C for 24 h. The methods used for fatty acid extraction, methyl ester preparation, and methyl ester separation by gas chromatography were described previously (28). The identification of the FAME profiles and their clustering have been described previously (12).

Py-MS. Cells were grown on Columbia blood agar for 24 to 48 h at 30°C (37°C for *P. larvae*). Preparation and pyrolysis mass spectrometry (Py-MS) of the bacterial samples (in quadruplicate for each strain) and normalization and cluster analysis of the data with GENSTAT statistical software (11) using the similarity coefficient of Gower (*S_G*) were performed as described previously (8, 12).

Phenotypic characterization and numerical analysis. Strains were grown on Columbia blood agar at 30°C (37°C for *P. larvae*) and characterized by the methods of Logan and Berkeley (18), using Analytab Products (API) tests and other phenotypic observations (with the exception of anaerobic growth), and the data were recorded, as described previously (12). The data were subjected to numerical taxonomic analysis using the GENSTAT 5 program (11). Similarities, with negative matches in the API tests included, were calculated by using *S_G* (17), and the results were examined by cluster analysis with the unweighted-pair group method using arithmetic averages (UPGMA) and principal-coordinate analysis (17). Ordination plots were rotated by using the Data Desk program.

DNA preparation. DNA for DNA-DNA binding experiments (see below) was extracted from 5 to 10 g (wet weight) of cells harvested from Roux flasks containing 130 ml of Columbia blood agar (for *P. larvae* strains and *P. pulvifaciens* LMG 6911^T) or 130 ml of nutrient agar (pH 7.4) supplemented with 1% (wt/vol) glucose (for the other strains). For DNA preparation, a method slightly modified from that of Marmur (19) was used, as described previously (31).

For the amplified ribosomal DNA restriction analysis and AFLP analysis (see below), total genomic DNA was purified by using a modification of the method of Pitcher et al. (23), as described previously (12). However, for extraction of DNA from *P. pulvifaciens* strains, both an additional proteinase K treatment at 37°C for 1 h at a final concentration of 250 µg/ml in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) after the lysozyme step and addition of NaCl to a final concentration of 1 M after the lysis of the cells with guanidium-EDTA-Sarkosyl reagent were necessary.

Percent DNA-DNA binding. DNA-DNA binding reactions of representative strains of each species were determined spectrophotometrically by using the initial renaturation method of De Ley et al. (4) and expressed as percentages, as described previously (30).

DNA base composition. Mean guanine-plus-cytosine (G+C) contents were determined by using the thermal denaturation method (5) and the equation of Marmur and Doty (20) as modified by De Ley (3).

Amplified ribosomal DNA restriction analysis (ARDRA). Enzymatically amplified genes encoding 16S rRNAs (16S rDNAs) were obtained and analyzed as described previously (12), with two modifications. First, the restriction enzyme

FIG. 1. (a) Dendrogram based on the UPGMA clustering of correlation coefficients (S_D) of normalized composite 16S rDNA restriction profiles (ARDRA) of *P. larvae* and *P. pulvifaciens* strains obtained with the restriction enzymes *Hae*III, *Dpn*II, *Rsa*I, *Bfa*I, and *Tru*9I. *P. alvei*, *P. validus*, *P. polymyxa*, and *P. pabuli* were used as out-groups. (b) Normalized computer profiles of *P. larvae* and *P. pulvifaciens* 16S rDNAs restricted with the indicated enzymes. Abbreviations: L, *P. larvae*; P, *P. pulvifaciens*. Lane M, molecular weight marker.

*Mse*I was replaced by its isoschizomer *Tru*9I (Boehringer GmbH, Mannheim, Germany), and second, UV-illuminated gels were documented with the Foto/Analyst Visionary Benchtop Digital Documentation System (Fotodyne, Hartland, Wis.) equipped with a charge-coupled device camera with a photoreceptor array (768 by 494 pixels) for registration of the patterns. The captured gel images were then digitized and stored in a personal computer as 8-bit TIFF files with an IRIS Video Digitizer card and Colorvision software (Inside Technology, Amersfoort, The Netherlands).

AFLP analysis. AFLP is a novel high-resolution DNA fingerprinting technique (28a) based on the selective amplification of restriction fragments (32). The technique consists of digesting total cellular DNA with two restriction enzymes, ligation of restriction half-site-specific adapters to the fragments, and using the ligated adapters as binding sites for the PCR primers. Only a subset of fragments will be amplified because the primers contain at their 3' ends one or more selective nucleotides extending beyond the restriction site so that under stringent PCR conditions only perfectly matched primers will lead to DNA synthesis. We followed essentially all the procedures recommended by Zabeau and Vos (32) except that 1 µg of DNA was digested with 10 U of the restriction enzymes *Eco*RI and *Mse*I (Pharmacia Biotech Benelux, Roosendaal, The Netherlands) in

a reaction volume of 30 µl. After digestion, *Eco*RI half-site- and *Mse*I half-site-specific adapters were ligated to the restriction fragments at final concentrations of 0.04 and 0.4 µM, respectively. The adapter design and sequences have been described previously (32). Selective amplification with primers E02 (5'-GACT GCGTACCAATTCC) and M01 (5'-GATGAGTCCTGAGTAAa) (selective nucleotides are indicated in lowercase) was done with a Perkin-Elmer 9600 thermal cycler as described previously (32) with the following modifications: for each reaction mixture, 20 ng of template DNA (restriction fragments with ligated adapters), 6.25 ng of ³²P-labeled E02 primer, 30 ng of unlabeled M01 primer, and 0.6 U of *Euro*Taq DNA polymerase (Eurogentec, Seraing, Belgium) were used, and in the temperature program the last 12 cycles were omitted because of the lesser complexity of bacterial genomes. PCR products were electrophoresed on 5% polyacrylamide–8.3 M urea sequencing gels and visualized by autoradiography. Banding patterns on the autoradiograms were captured with a RayVen RSU1 densito scanner (X-Ray Scanner Corporation) and analyzed with GelCompar software (Applied Maths, Kortrijk, Belgium) using the Dice coefficient (S_D) (22) and the UPGMA clustering algorithm.

RAPD. PCRs for random amplified polymorphic DNA (RAPD) analysis and data interpretations were carried out as described previously (12) except that two

FIG. 2. Normalized computer profiles of AFLP patterns of *P. larvae* and *P. pulvifaciens* strains using selective PCR primers E02 and M01. *Paenibacillus validus* and *P. pabuli* were used as out-groups in the numerical analysis presented in a dendrogram based on the UPGMA clustering of correlation coefficients (S_D). Abbreviations: L, *P. larvae*; P, *P. pulvifaciens*; V, *P. validus*; Pa, *P. pabuli*.

FIG. 3. Normalized computer profiles of protein patterns (PAGE) of *P. larvae* and *P. pulvifaciens* strains. *P. alvei*, *P. validus*, and *P. polymyxa* were used as out-groups. The zone (to the right of the arrow) which contains the high-molecular-weight band in the *P. pulvifaciens* patterns was omitted from the numerical analysis presented in a dendrogram based on the UPGMA linkage of correlation coefficients (r). Abbreviations: A, *P. alvei*; L, *P. larvae*; P, *P. pulvifaciens*; V, *P. validus*; Po, *P. polymyxa*.

TABLE 2. Comparison of fatty acid profiles of three *P. larvae* and three *P. pulvificiens* strains after 24 h of growth on Columbia blood agar at different growth temperatures as measured by gas chromatographic analysis of their methyl esters

Species and growth temp	Mean % (range) ^a										Summed feature 4 ^b	Summed feature 6 ^c
	14:0 iso	14:0	15:0 iso	15:0 anteiso	15:0	16:0 iso	16:0	17:0 iso	17:0 anteiso	17:0		
<i>P. pulvificiens</i>												
	0.5 (0.5-0.5)	1.6 (1.6-1.6)	15.5 (15.3-15.7)	42.5 (42.6-52.8)	2.7 (2.4-3.3)	4.4 (3.9-4.8)	9.2 (9.1-9.3)	6.7 (6.6-6.8)	14.6 (14.2-14.9)	0.8 (0.7-1.0)	0.8 (0.0-1.1)	0.3 (0.0-0.6)
	0.3 (0.0-0.4)	0.9 (0.8-1.0)	14.3 (14.0-14.7)	39.6 (38.6-40.8)	2.5 (2.0-3.6)	6.2 (5.7-6.5)	7.6 (6.6-8.5)	7.5 (7.1-7.8)	18.9 (18.3-19.2)	0.8 (0.7-1.1)	0.9 (0.0-1.4)	0.5 (0.4-0.6)
<i>P. larvae</i>												
	1.5 (1.2-1.9)	1.7 (1.3-1.9)	18.0 (16.6-21.3)	37.4 (33.5-40.8)	1.8 (1.0-2.6)	8.0 (6.5-9.1)	9.7 (7.7-11.0)	7.8 (6.6-10.5)	11.6 (10.3-14.0)	0.2 (0.0-0.6)	1.7 (1.4-1.8)	0.3 (0.0-0.5)
	0.8 (0.7-0.9)	0.8 (0.8-0.8)	12.2 (10.7-13.7)	32.6 (30.9-35.7)	1.8 (1.1-2.3)	10.4 (8.6-12.2)	9.1 (8.3-9.7)	9.6 (9.2-10.5)	20.0 (19.3-21.2)	0.9 (0.5-1.3)	1.8 (1.4-2.2)	0.1 (0.0-0.4)

^a Values in parentheses are the minimal and maximal values within each group of strains.^b Summed feature 4, 15:0 iso 2OH and/or 16:1 ω7t (not separated by the MIS).^c Summed feature 6, 18:2 ω6,9c and/or 18:0 anteiso (not separated by the MIS).^d Trace amounts (<0.3% of the total fatty acids) of 10:0 present.^e Trace amounts (<0.3% of the total fatty acids) of 13:0 iso and 18:0 present.TABLE 3. Phenotypic characteristics of *P. larvae* and *P. pulvificiens* strains^{a,b}

Characteristic or test	<i>P. larvae</i>	<i>P. pulvificiens</i>
Motility	v	v
Ellipsoidal spores	+	+
Central and subterminal spores	+	+
Swollen sporangia	+/-	+/-
Anaerobic growth	+	+
Thiamine requirement	+	-
Esculin hydrolysis	+	+
Arginine dihydrolase	-	+
Casein hydrolysis	+	+
Citrate utilization	-	-
Hydrogen sulfide production	-	-
Indole production	-	-
Gelatin liquefaction	+	+
Nitrate reduction	v	v
ONPG ^c	-	-
Urease	-	-
Voges-Proskauer	v	v
Acid from ^d :		
<i>N</i> -Acetylglucosamine, D-glucose, glycerol, D-mannose, ribose, and D-trehalose	+	+
Mannitol	-	+
Salicin	+	-
D-Fructose, D-galactose, methyl-D-glucoside, and D-tagatose	v	v

^a With the exception of anaerobic growth, thiamine requirement, and microscopic properties, all characters were determined by using API 20E and 50CHB System tests.^b +, 90% or more of the strains positive; -, 90% or more of the strains negative; v, variation between strains; +/-, variation within strains.^c ONPG, *o*-nitrophenyl-β-D-galactopyranoside.^d Acid was not produced from adonitol, amygdalin, D- and L-arabinose, D- and L-arabitol, arbutin, D-cellobiose, dulcitol, erythritol, D- and L-fucose, gentiobiose, D-gluconate, 2-keto-D-gluconate, 5-keto-D-gluconate, glycogen, *meso*-inositol, inulin, lactose, D-lyxose, maltose, D-melibiose, D-melezitose, methyl-D-mannoside, methyl-D-xyloside, D-raffinose, L-rhamnose, D-sorbitol, L-sorbose, starch, sucrose, D-turanose, xylitol, and D- and L-xylose.

additional primers were used: 24671 (5'-ATCTGCAGCTGAACGGTCTGG-3') and a universal primer, 5'-TATGTAAAACGACGGCCAGT-3'.

RESULTS AND DISCUSSION

ARDRA. By numerical analysis of the ARDRA banding patterns of different *Bacillus* rRNA group 3 species (reclassified as *Paenibacillus* spp. [2]), the six *P. larvae* and *P. pulvificiens* strains studied were clustered at more than 90% similarity. This *P. larvae*-*P. pulvificiens* cluster was clearly separated from other *Paenibacillus* species such as *P. alvei*, *Paenibacillus pabuli*, *P. polymyxa*, and *P. validus* (Fig. 1a). In Fig. 1b, the restriction patterns with all restriction enzymes used are shown for *P. larvae* and *P. pulvificiens* strains.

The high degree of similarity of the rDNA restriction patterns is supported by the nearly identical 16S rDNA sequences of the *P. larvae* type strain and *P. pulvificiens* NCDO 1141 (1). Because restriction analysis of enzymatically amplified rDNA can be used to delineate species (e.g., see references 12, 13, and 26), the ARDRA data strongly suggest that the two *Paenibacillus* species should be united at the species level.

AFLP. The AFLP patterns of the *P. larvae* and *P. pulvificiens* strains exhibited a high (>80%) overall similarity by numerical analysis (Fig. 2). The levels of similarity with the out-groups *P. pabuli* and *P. validus* were much lower. However, the *P. larvae* strains on the one hand and the *P. pulvificiens* strains on the other grouped in two distinct clusters, with respective internal similarities of 88 and 95% (Fig. 2), which indicates that AFLP

FIG. 4. (a) Dendrogram based on the UPGMA clustering of correlation coefficients (S_G) of Py-MS data for *P. larvae* and *P. pulvifaciens* strains. *P. alvei*, *P. validus*, and *P. polymyxa* were used as out-groups. (b) Canonical variates (CV) plot of Py-MS data showing the distribution of *P. larvae* and *P. pulvifaciens* strains and the out-group species. ●, *P. larvae*; +, *P. pulvifaciens*; *, *P. polymyxa*; □, *P. alvei*; x, *P. validus*.

profiles allowed discrimination at the infraspecific level. AFLP is very useful in the characterization of bacteria at the subgeneric level and allows differentiation within the range biovar to genomic species (15). The AFLP data thus not only support but also extend the rDNA analysis by indicating the existence of two subgroups.

SDS-PAGE. Numerical analysis of complete PAGE data of the organisms grown on nutrient agar at 30°C exhibited a clustering of the *P. pulvifaciens* and *P. larvae* strains at 64% similarity (not shown). This low clustering level was due to the presence of a dense protein band in the upper part of the profiles of *P. pulvifaciens* protein extracts (Fig. 3). When the zone containing this band and all other bands with molecular weights of more than 1.3×10^5 was left out of the numerical analysis, all *P. larvae* and *P. pulvifaciens* strains clustered above 82% similarity, with type strains (including duplicate subcultures) even linking at the 90% similarity level. This was well above the levels of linkage with the nearest neighbors, *P. alvei*, *P. polymyxa*, and *P. validus*. Different growth conditions (Columbia blood agar or nutrient agar with glucose and 30 or 37°C incubation temperature) did not have an important effect on the similarity values: with cultures from blood agar, the simi-

larity between the *P. larvae* and the *P. pulvifaciens* protein patterns was 87%. The PAGE data thus support the rDNA data. Furthermore, as the dense protein band was present in only the *P. pulvifaciens* patterns (Fig. 3), two distinct infraspecific groups corresponding to the two subclusters found with the AFLP technique (see above) could be discriminated.

FAME. It was not possible to obtain growth of *P. larvae* on TSA plates in 24 h at 28°C (the conditions recommended by the MIS), whereas *P. pulvifaciens* grew confluent. However, *P. larvae* grew (although rather weakly) at 37°C within 48 h, but not at 28°C, when TSA was supplemented with thiamine. As could be expected, unsatisfactory FAME profiles were obtained following cultivation under these inappropriate growth conditions. Confluent growth of *P. larvae* was obtained after 24 h on Columbia blood agar at 37 or 28°C. The revealed FAME patterns (Table 2) of *P. larvae* and *P. pulvifaciens* were very similar, and all strains clustered below the Euclidian distance of 10 (not shown), supporting the idea of a single-species status. These FAME data are in good agreement with the observations of Drobníková et al. (7), who also found that *P. larvae* and *P. pulvifaciens* exhibit similar fatty acid profiles.

Py-MS. On the phenogram shown in Fig. 4a, the strains of *P.*

FIG. 5. Revealed RAPD patterns of *P. larvae* and *P. pulvifaciens* strains with primer 24671. *P. alvei*, *P. polymyxa*, and *P. validus* were used as out-groups. Identical profiles were observed for the duplicate type strains of *P. larvae* (LMG 9820 and LMG 15969) and *P. pulvifaciens* (LMG 6911 and LMG 15974). Lane M, molecular weight marker.

larvae and *P. pulvifaciens* (including duplicate type strains) grouped together in a single cluster with an overall similarity of 83%, whereas the similarity of this cluster to that including the three out-group species was only 50%. It is generally recognized that representation of multidimensional taxonomic structures by two-dimensional phenograms may give distorted impressions of relationships (17); the cluster analysis procedure used here produces phenograms with special properties, so that careful interpretation with reference to canonical variate analysis or other nonhierarchical methods is necessary (10). The phenogram in Fig. 4a did not, for example, indicate the high degree of similarity found between the duplicated type strains of *P. larvae* and *P. pulvifaciens*. In the similarity matrix (not shown) on which the phenogram was based, each of the two pairs of type strains was 93% similar. Examination of a canonical variate analysis plot of the data (Fig. 4b) shows that the *P. larvae* and *P. pulvifaciens* strains fell into two close but discrete groups, distant from the strains representing the three out-group species.

RAPD patterns. The banding patterns obtained with primer 24671 were more complex than those obtained with the other two primers (the universal primer mentioned in Materials and Methods and primer 42423 [12]). The profiles of the strains of *P. larvae* and *P. pulvifaciens* were very similar and clearly different from those of the out-group organisms (Fig. 5). Numerical analysis of the data (not shown) revealed similarities of 80 to 95% for the *P. larvae* and *P. pulvifaciens* strains, demonstrating their close relatedness but showing nonetheless that they fell into two distinct groups. Similar conclusions could be drawn from RAPD data obtained with the other two primers (not shown).

Biochemical, physiological, and morphological characters. The four duplications (*P. larvae* LMG 9820^T and LMG 15969^T, *P. pulvifaciens* LMG 6911^T [twice] and LMG 15974^T, and *P.*

validus 11161^T [twice]) showed similarities ranging from 95 to 98%. This implies an overall test error of 3.25% ($P \sim 1.625\%$), which is acceptably low (17); although many results such as those from the API tests were scored quantitatively, which might increase the apparent error, this would be balanced to some extent by *P. larvae* and *P. pulvifaciens* strains giving many negative reactions in these tests. Both nonhierarchical (principal-coordinate) and hierarchical (cluster analysis) methods were used to arrange the strains, as the former approach gives a less distorted view of relationships between groups, while the latter gives a more faithful representation of within-group relationships (17). A rotated principal-coordinate plot of vectors 1 through 3, which together accounted for 89.6% of the variation in the data (vector 1, 60.9%; vector 2, 20.7%; and vector 3, 8.0%), is shown in Fig. 6a. When the plot was rotated, the strains of *P. larvae* and *P. pulvifaciens* appeared as a stable pattern of two distinct but adjacent, elongated groups; the strains representing other species showed more-diffuse species structures, and the strains of *P. polymyxa* and *P. pabuli* could not be separated. A similar picture emerged from the phenogram (Fig. 6b): strains of *P. larvae* and *P. pulvifaciens* were recovered in two distinct species clusters which formed at 95 and 94% S_G , respectively, and these two clusters merged at 89% S_G . Strains of the other species clustered at lower similarity levels of between 84 and 86% S_G . Although the two strains of *P. polymyxa* joined at 94% S_G , reliable separation from *P. pabuli* by these phenotypic tests is not possible, as the ordination plot (Fig. 6a) showed. The strains of *P. larvae* and *P. pulvifaciens* showed similar patterns of reactions overall but were distinguishable by API tests (Table 3) and could be separated from other species of *Paenibacillus* (Table 4).

Moles percent G+C and DNA-DNA binding studies. The type strains of *P. larvae* and *P. pulvifaciens* showed a DNA-DNA relatedness of 90%. Two other strains of the two species

FIG. 6. (a) Rotated plot of the first three vectors of a principal-coordinate analysis of 86 phenotypic characters of 19 *Paenibacillus* strains. (b) Phenogram based on an UPGMA analysis of similarities of 19 *Paenibacillus* strains based on 86 phenotypic characters. Strain numbers are LMG numbers; d, duplicate strain.

(LMG 14428 and LMG 14426) showed a relatedness of 81% to each other and 99 and 95% relatedness, respectively, to their type strains (Table 5). These data confirm the synonymy of these two taxa at the species level. They are in disagreement with the low DNA relatedness values of 25 to 36% between *P. pulvifaciens* strains and the *P. larvae* type strain reported by Nakamura (21). Furthermore, the G+C content of 42.2 to 42.3 mol% (Table 5) for the genomic DNA of *P. larvae* was also in disagreement with the 50 mol% G+C for the type strain of *P. larvae* determined by Nakamura with the buoyant-density method (21). The different methods used for the G+C content determination may only partially explain this difference in reported DNA base composition. Unfortunately, Nakamura (21) included only one representative of *P. larvae* in his study. In our study, we have analyzed several strains of both species, including new American Type Culture Collection subcultures of both type strains (Table 1), to reconfirm our data. The AFLP (not shown), PAGE, RAPD, and Py-MS profiles and phenotypic characters of the duplicate type strains were identical, which is in favor of the authenticity of the type strains used in this polyphasic study.

Conclusion. In a polyphasic analysis comprising morphological and biochemical tests, a variety of chemotaxonomic and genomic fingerprints, and DNA relatedness measurements, all eight strains of *P. larvae* and *P. pulvifaciens* showed high levels of similarity. Therefore, we conclude that *P. pulvifaciens* is a later synonym of *P. larvae*. At the infraspecific level, however, phenotypic and genotypic differentiation into two subspecies is

possible, and this is consistent with the different pathologies of *P. larvae* and *P. pulvifaciens*. In view of these considerations, we propose reclassification of *P. larvae* strains as *P. larvae* subsp. *larvae* and of *P. pulvifaciens* strains as *P. larvae* subsp. *pulvifaciens*. An emended description of *P. larvae* and descriptions of *P. larvae* subsp. *larvae* and *P. larvae* subsp. *pulvifaciens* based on our data and the data of Gordon et al. (9), Nakamura (21), and Ash et al. (2) are given.

Emended description of *Paenibacillus larvae*. *Paenibacillus larvae* (lar'vae. L. n. larva ghost; M.L. n. larva, a larva; M.L. gen. n. larvae, of a larva). Round-ended, straight and sometimes curved rods, 0.5 μ m wide by 1.5 to 6 μ m long, occurring singly and in chains and filaments; some strains are motile. Sporangia are often sparse in vitro, and the ellipsoidal, central to subterminal spores, which may swell the sporangia (Fig. 7), are often found free.

Isolated from diseased honeybee (*A. mellifera*) larvae. Facultatively anaerobic, catalase negative or weak, delayed positive. Growth occurs at temperatures between 20 to 25 and 40°C. The organisms grow well on Columbia blood agar containing 5% horse blood, which may become discolored or partially hemolyzed, and on MYPGP agar (described by Dingman and Stahly [6]).

When tested for with the API 20E strip, gelatin and casein are hydrolyzed; *o*-nitrophenyl- β -D-galactopyranoside is not hydrolyzed; citrate is not utilized; hydrogen sulfide, urease, and indole are not produced; and weak reactions for Voges-Proskauer and nitrate reduction are detectable in some strains.

TABLE 4. Characteristics for differentiating *P. larvae* and *P. pulvifaciens* from related *Paenibacillus* species

Characteristic ^a	<i>P. larvae</i>	<i>P. pulvifaciens</i>	<i>P. alvei</i>	<i>P. validus</i>	<i>P. polymyxa</i>
Anaerobic growth	+	+	+	—	+
Arginine dihydrolase	—	+	—	—	—
Indole production	—	—	+	—	—
Gelatin liquefaction	+	+	+	—	+
ONPG ^b	—	—	+	—	+
Acid from:					
<i>N</i> -Acetylglucosamine	+	+	+	—	—
Maltose	—	—	+	+	+
Starch	—	—	+	v ^c	+
Sucrose	—	—	v	+	+
Mannitol	—	+	—	+	+
Salicin	+	—	v	—	+
D-Xylose	—	—	—	+	+
Gas from carbohydrates	—	—	—	—	+

^a With the exception of anaerobic growth, all characteristics were determined by using API 20E and 50CH (with API CHB suspension medium) System tests; anaerobic growth data are from references 9 and 12.

^b ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

^c v, between-strain variation.

In the API 50CH gallery, using the API CHB suspension medium, acid is produced from *N*-acetylglucosamine, D-glucose, glycerol, D-mannose, ribose, and D-trehalose; esculin is hydrolyzed; acid is not produced from adonitol, amygdalin, D- and L-arabinose, D- and L-arabitol, arbutin, D-cellobiose, dulcitol, erythritol, D- and L-fucose, gentiobiose, D-gluconate, 2-keto-D-gluconate and 5-keto-D-gluconate, glycogen, *meso*-inositol, inulin, lactose, D-lyxose, D-maltose, D-melibiose, D-melezitose, methyl-D-mannoside, methyl-D-xyloside, D-raffinose, L-rhamnose, D-sorbitol, L-sorbose, starch, sucrose, D-turanose, xylitol, and D- and L-xylose. Different strains or subspecies give different results with D-fructose, D-galactose, mannitol, methyl-D-glucoside, salicin, and D-tagatose, as well as for the arginine dihydrolase reaction.

When characterized by the methods of Gordon et al. (9), strains will grow in media containing 0.001% lysozyme or 2% NaCl but not 5% NaCl; they are positive for decomposition of

TABLE 5. G+C contents of *P. larvae* and *P. pulvifaciens* strains and DNA-DNA binding data within and between the two species^a

Species	LMG no.	% G+C	% DNA binding with strain:				
			6911 ^T	14427	14428	9820 ^T	14426
<i>P. pulvifaciens</i>	6911 ^T	43.3	100				
	14427	42.9	100	100			
	14428	42.0	99	100	100		
<i>P. larvae</i>	9820 ^T	42.3	90			100	
	14426	42.2		81		95	100
<i>P. validus</i>	14666	52.3		17			
	14019	50.9				4	

^a *P. validus* was used as an out-group. Data are means of at least two experiments.

casein and gelatin and for acid production from glucose and trehalose; results vary for acid production from mannitol; most strains reduce nitrate to nitrite; they are negative for the Voges-Proskauer test, growth at pH 5.7, starch hydrolysis, decomposition of tyrosine, deamination of phenylalanine, formation of dihydroxyacetone and indole, and acid production from arabinose and xylose.

The following fatty acids are present after 24 h of growth on Columbia blood agar for more than 1% of the total fatty acid content: 14:0, 15:0 iso, 15:0 anteiso, 16:0 iso, 16:0, 17:0 iso, and 17:0 anteiso. The internal DNA relatedness of the strains is above 70%. ARDRA using *Hae*III, *Dpn*II, *Rsa*I, *Bfa*I, and *Tru*9I reveals similar patterns.

Description of *Paenibacillus larvae* subsp. *larvae*. The morphological, chemotaxonomic, and general characters of *Paenibacillus larvae* subsp. *larvae* are the same as those given above for the species. Causes American foulbrood disease of honeybee (*A. mellifera*) larvae. Optimum temperature is 35 to 37°C; no growth at 20°C. Thiamine is required for growth. Will not grow in nutrient broth on repeated subculture. After 2 to 4 days on Columbia blood agar, colonies are small (<1 mm in diameter), regular, glossy, butyrous, and greyish or discolored with blood pigments. Catalase negative. Arginine dihydrolase not produced; acid produced from salicin (may be weak); no

FIG. 7. Photomicrographs of sporangia and vegetative cells of *P. larvae* subsp. *larvae* LMG 9820^T viewed by phase-contrast microscopy; sporangia are swollen or unswollen with central to subterminal ellipsoidal spores. Bar, 2 μm.

acid from mannitol in API 50CH gallery. When tested by the method of Gordon et al. (9), most strains do not produce acid from mannitol, and nitrate reduction varies between strains. The G+C content of the type strain is 42.3 mol% (renaturation method). The type strain is ATCC 9545 (LMG 9820 and LMG 15969). The 16S rDNA sequence of ATCC 9545^T is deposited in the EMBL Data Library with assigned accession no. X60619 (1).

Description of *Paenibacillus larvae* subsp. *pulvificiens*. *Paenibacillus pulvificiens* (pul.vi.fá.ciens. L. n. *pulvis*, powder, dust; L. pres. part. *faciens*, making; M.L. adj. *pulvificiens*, making powder or dust). The morphological, chemotaxonomic, and general characters of *P. larvae* subsp. *pulvificiens* are the same as those given above for the species. Causes powdery scale of honeybee (*A. mellifera*) larvae. Optimum growth temperature, 28 to 30°C; grows also at 20°C. Will grow in nutrient broth on repeated subculture. Grows on routine media such as nutrient agar, as well as on Columbia blood agar, producing unpigmented or yellow-orange-pigmented, smooth, glossy, regular, butyrous to sticky colonies 1 to 3 mm in diameter in 2 to 4 days at 30 or 37°C. Catalase negative or weak, delayed positive. Arginine dihydrolase is produced; nitrate is reduced to nitrite; acid is produced from mannitol but not from salicin. The average G+C content is 42.0 to 43.3 mol% (renaturation method) or 44 mol% (buoyant-density method). The type strain is NRRL B-3685 (LMG 6911, LMG 15974, ATCC 13537, and NCIMB 11201). The 16S rDNA sequence of the strain NCDO 1141 is deposited in the EMBL Data Library with assigned accession no. X60636 (1).

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