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Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation

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A polyphasic taxonomic study of the two subspecies of *Paenibacillus larvae*, *Paenibacillus larvae* subsp. *larvae* and *Paenibacillus larvae* subsp. *pulvifaciens*, supported the reclassification of the subspecies into one species, *Paenibacillus larvae*, without subspecies separation. Our conclusions are based on the analysis of six reference strains of *P. larvae* subsp. *pulvifaciens* and three reference strains and 44 field isolates of *P. larvae*. subsp. *larvae*. The latter originated from brood or honey of clinically diseased honey bee colonies or from honey of both clinically diseased and asymptomatic colonies from Sweden, Finland and Germany. Colony and spore morphology, as well as the metabolism of mannitol and salicin, did not allow a clear identification of the two subspecies and SDS-PAGE of whole-cell proteins did not support the subspecies differentiation. For genomic fingerprinting, repetitive element-PCR fingerprinting using ERIC primers and PFGE of bacterial DNA were performed. The latter method is a high-resolution DNA fingerprinting method proven to be superior to most other methods for biochemical and molecular typing and has not previously been used to characterize *P. larvae*. ERIC-PCR identified four different genotypes, while PFGE revealed two main clusters. One cluster included most of the *P. larvae* subsp. *larvae* field isolates, as well as all *P. larvae* subsp. *pulvifaciens* reference strains. The other cluster comprised the pigmented variants of *P. larvae* subsp. *larvae*. 16S rRNA gene sequences were determined for some strains. Finally, exposure bioassays demonstrated that reference strains of *P. larvae* subsp. *pulvifaciens* were pathogenic for honey bee larvae, producing symptoms similar to reference strains of *P. larvae* subsp. *larvae*. In comparison with the type strain for *P. larvae* subsp. *larvae*, ATCC 9545^T, the *P. larvae* subsp. *pulvifaciens* strains tested were even more virulent, since they showed a shorter LT_{100}. An emended description of the species is given.

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Abbreviations: AFB, American foulbrood; MRPs, macrorestriction profiles; rep-PCR, repetitive element-PCR fingerprinting.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Paenibacillus larvae* strains 03-525, 00-1163, 02-130 and 03-189 are DQ079620–DQ079623, respectively.

A table giving the CCUG accession numbers for the *P. larvae* field strains used in the study and figures showing colony and spore morphology are available as supplementary material in IJSEM Online.

INTRODUCTION

American foulbrood (AFB), a fatal bacterial disease of honey bee brood, has spread worldwide (Matheson, 1993) and is a considerable threat to beekeeping in many countries as it is highly contagious and often lethal to managed bee colonies. The clinical symptoms of AFB are typical, with the brown, viscous larval remains forming a ropy thread when drawn out with a matchstick. The decaying brood desiccates into hard scales, tightly adhering to the walls of the cells,

consisting of millions of bacterial spores which are the infectious stage of the pathogen.

A rare condition, known as powdery scale disease of honey bee brood, was described by Katznelson (1950). He isolated a spore-forming bacterium from powdery decays of dead larvae, considered the aetiological agent of powdery scale disease. However, later he concluded that this organism was not pathogenic to honey bees (Katznelson & Jamieson, 1951). Yet Hitchcock *et al.* (1979) provided evidence that the organism isolated by Katznelson (1950) is pathogenic to honey bee larvae. The acute symptoms of this disease have never been properly described, although findings of powdery scales were later reported by Gilliam & Dunham (1978), who isolated bacterial strains phenotypically related to those isolated by Katznelson (1950).

The causative agents of AFB and powdery scale disease were originally described as *Bacillus larvae* (White, 1906) and *Bacillus pulvifaciens* (Katznelson, 1950), respectively. Both species were later transferred to the genus *Paenibacillus* (Ash *et al.*, 1993). In 1996, a polyphasic taxonomic approach, including DNA–DNA binding studies, revealed high levels of similarity between the two species that did not support the classification of two separate species. However, infraspecific differences and the contrasting described pathologies led to the reclassification of *P. larvae* strains as *P. larvae* subsp. *larvae* and *P. pulvifaciens* strains as *P. larvae* subsp. *pulvifaciens* (Heyndrickx *et al.*, 1996).

Besides differences in pathogenicity, *P. larvae pulvifaciens* is described as differing from *P. larvae larvae* by having a striking orange-pigmented colony morphology not seen with *P. larvae larvae* (Heyndrickx *et al.*, 1996), by showing differences in external spore structure (Ludvik *et al.*, 1993) and by the production of acid from mannitol, but not from salicin (Heyndrickx *et al.*, 1996). The presence of orange-pigmented colonies is considered to be one of the most obvious hallmarks of *P. larvae pulvifaciens*, often leading to the classification of such strains as subspecies *pulvifaciens*. However, although orange-pigmented colonies are said to be characteristic for *P. larvae pulvifaciens* (Heyndrickx *et al.*, 1996), orange-pigmented colonies isolated from diseased brood with symptoms of AFB have been reported (Drobnikovà *et al.*, 1994) and demonstrated to represent a particular genotype of *P. larvae larvae* (Neuendorf *et al.*, 2004). Recently, a PCR method for the differentiation of the two subspecies of *P. larvae* was developed, based on molecular subtyping performed with repetitive element-PCR (rep-PCR) using ERIC primers (Alippi *et al.*, 2004). The results of Neuendorf *et al.* (2004), however, suggest strongly that the rep-PCR described by Alippi *et al.* (2004) might be specific for a certain genotype of *P. larvae* rather than being subspecies-specific.

There are several studies on the relatedness and classification of *P. larvae larvae* and *P. larvae pulvifaciens*, most of them using strain DSM 3615^T or synonymous strains as a reference strain for *P. larvae pulvifaciens* (Alippi *et al.*, 2002; Ash *et al.*, 1993; Heyndrickx *et al.*, 1996; Lauro *et al.*, 2003). As this isolate has been demonstrated to be indistinguishable from reference strains of *P. larvae larvae* (Kilwinski *et al.*, 2004), the validity of these studies can be questioned.

The aim of this study was to establish whether the classification of two subspecies within the species *P. larvae* is justified.

METHODS

Bacterial isolates. Forty-four field isolates of *P. larvae* from Sweden, Finland and Germany were studied (Table 1). Supplementary Table S1 (available in IJSEM Online) details the 39 strains that have been deposited at the Swedish Culture Collection, University of Gothenburg, Sweden (CCUG). Two German field isolates, 03-522 ger and 03-525 ger, were deposited at the DSMZ as DSM 16115 and DSM 16116, respectively. *P. larvae larvae* strains CCUG 28515^T, ATCC 9545^T and DSM 7030^T were used as reference strains. The *P. larvae pulvifaciens* reference strains used in this study were CCUG 7427 (=NCDO 1121), ATCC 49843^T, DSM 3615^T, LMG 6911^T, LMG 16247 (=Katznelson 754=NRRL B-3687) and LMG 16252 (=Gilliam 2=NRRL NRS-1684) (Table 1).

Isolation and identification. Swedish material from larvae with acute symptoms of AFB was cultivated on MYPGP-agar plates as described by Nordström & Fries (1995). MYPGP-agar contained (l^{-1}), 10 g Müller–Hinton broth, 15 g yeast extract, 3 g K_2HPO_4, 1 g sodium pyruvate, 20 g agar and 2 g glucose. Finnish larvae samples and undiluted honey samples were plated on to T-HCl-YGP agar plates (Steinkraus & Morse, 1996). T-HCl-YGP agar contained (l^{-1}), 15 g yeast extract (Difco), 1 g pyruvic acid (Sigma), 200 ml 0·1 M Tris/HCl, pH 7·0, 20 g agar, 40 ml 10% glucose and 0·6 ml malidixic acid. Honey was boiled for 15 min before culturing. Plates were incubated at 35 °C for 10 days. German samples were processed as described by Neuendorf *et al.* (2004).

Colonies with a *P. larvae*-like morphology (whitish, somewhat transparent and glistening appearance or orange–brown, non-transparent appearance) were further analysed for catalase activity, mannitol fermentation and/or formation of giant whips. For the catalase test, part of the colony was transferred to a microscope slide and mixed with a drop of 30 % H_2O_2. Production of bubbles is indicative for catalase activity, absence of bubbles indicates lack of activity. Strains were tested by the method of Gordon *et al.* (1973) for the production of acid from mannitol and/or by the method of Plagemann (1985) for the formation of giant whips. In addition, all but three field isolates have already been classified by the CCUG according to colony morphology, catalase activity and API tests (Heyndrickx *et al.*, 1996). Thirteen field isolates, some of them isolated from diseased brood showing clinical symptoms of AFB, have been preliminarily diagnosed as *P. larvae pulvifaciens* (Table 1).

rep-PCR analysis. Preparation of bacterial DNA for fingerprinting and subsequent rep-PCR analysis was performed essentially as previously described (Genersch & Otten, 2003). In brief, *P. larvae larvae* DNA suitable for rep-PCR DNA fingerprinting was isolated from colonies grown on culture plates using 6 % InstaGene matrix (Bio-Rad) following the manufacturer's instructions. The DNA sequences of the primers used for DNA fingerprinting were 5′-ATGTAAGCT-CCTGGGGATTCAC-3′ (ERIC1R) and 5′-AAGTAAGTGACTGGG-GTGAGCG-3′ (ERIC2) (Versalovic *et al.*, 1994).

PCR were carried out in a final volume of 25 µl consisting of 1 × reaction buffer (Qiagen) and final concentrations of 2·5 mM $MgCl_2$, 250 µM dNTPs (dATP, dCTP, dGTP, dTTP), 10 µM primer and 0·3 U

Table 1. *P. larvae* strains and overview of methods applied for characterization

ERIC, group identified with rep-PCR with ERIC-primers; PFGE, *Not*I restriction followed by PFGE; 16S rRNA, 16S rRNA gene sequencing; PAGE, protein profiling via SDS-PAGE; Man/Sal, metabolism of mannitol and salicin as part of API tests; API ZYM, API ZYM tests for phosphatase activity. Methods used to characterize strains are indicated by +.

Strain	Source	Subspecies according to CCUG	ERIC	PFGE	16S rRNA	PAGE	Man/Sal	API ZYM
DSM 7030^T	DSMZ	*larvae*/ ref. strain	I		+		+	
ATCC 9545^T	ATCC	*larvae*/ ref. strain	I	+			+	+
CCUG 28515^T	CCUG	*larvae*/ref. strain	I			+		
ATCC 49843^T	ATCC	*pulvifaciens*/ref. strain	IV	+				+
DSM 3615^T	DSMZ	*pulvifaciens*/ref. strain	IV		+		+	
LMG 16247	LMG	*pulvifaciens*/ref. strain	IV	+		+		+
LMG 6911^T	LMG	*pulvifaciens*/ref. strain	IV				+	
LMG 16252	LMG	*pulvifaciens*/ref. strain	III	+		+		+
CCUG 7427	CCUG	*pulvifaciens*/ref. strain	IV			+		
33 swe	Diseased brood	*larvae*	I	+		+	+	+
96 swe	Diseased brood	*larvae*	I	+		+	+	+
36/97 swe	Diseased brood	*larvae*	I	+		+	+	+
159/97 swe	Diseased brood	*larvae*	I	+		+	+	+
176/97 swe	Diseased brood	*larvae*	I	+		+	+	+
87/98 swe	Diseased brood	*larvae*	I	+		+	+	+
207/99 swe	Diseased brood	*larvae*	I	+		+	+	+
78/00 swe	Diseased brood	*larvae*	I	+		+	+	+
166/00 swe	Diseased brood	*larvae*	I	+		+	+	+
185/00 swe	Diseased brood	*larvae*	I	+		+	+	+
233/00 swe	Diseased brood	*pulvifaciens*	II	+		+	+	+
35/01 swe	Diseased brood	*larvae*	I	+		+	+	+
150/01 swe	Diseased brood	*larvae*	I	+		+	+	+
26/02 swe	Diseased brood	*pulvifaciens*	II	+		+	+	+
118/02 swe	Diseased brood	*larvae*	I	+		+	+	+
144/02 swe	Diseased brood	*larvae*	I	+		+	+	+
97/03 swe	Diseased brood	*pulvifaciens*	II	+		+	+	+
127/03 swe	Diseased brood	*pulvifaciens*	II	+		+	+	+
57/04 swe	Diseased brood	*pulvifaciens*	II				+	
00-087 ger	Honey (dis. col.)	*larvae*	I	+		+	+	+
00-1163 ger	Honey (dis. col.)	*P. larvae*	II	+	+	+	+	+
02-130 ger	Honey (dis. col.)		I		+		+	
03-119 ger	Honey (dis. col.)	*larvae*	I	+		+	+	+
03-130 ger	Honey (dis. col.)	*larvae*	I				+	
03-159 ger	Honey (dis. col.)	*larvae*	I	+		+	+	+
03-189 ger	Honey (dis. col.)		I		+		+	
03-522 ger	Honey (dis. col.)	*pulvifaciens*	II	+		+	+	+
03-525 ger	Honey (dis. col.)		II		+		+	
888/97 fin	Honey	*larvae*	I	+		+	+	+
925/97 fin	Honey	*larvae*	I	+		+	+	+
1051/97 fin	Diseased brood	*larvae*	I	+		+	+	+
110/99 fin	Honey	*larvae*	I	+		+	+	+
992/99 fin	Honey	*larvae*	I	+		+	+	+
910/01 fin	Honey	*larvae*	I	+		+	+	+
1275/01 fin	Honey	*larvae*	I	+		+	+	+
1847/01 fin	Diseased brood	*larvae*	I	+		+	+	+
3264/02 fin	Honey	*larvae*	II	+		+		+
3284/02 fin	Honey	*pulvifaciens*	II	+		+	+	+
7774/03 fin	Honey	*pulvifaciens*	II			+	+	+
7789/03 fin	Honey	*pulvifaciens*	II	+			+	+

Table 1. cont.

Strain	Source	Subspecies according to CCUG	ERIC	PFGE	16S rRNA	PAGE	Man/Sal	API ZYM
7766/03 fin	Honey	*pulvifaciens*	II	+		+	+	+
8514/03 fin	Honey	*pulvifaciens*	II	+		+	+	+
8533/03 fin	Honey	*pulvifaciens*	II	+		+	+	+
8543/03 fin	Honey	*pulvifaciens*	II	+		+	+	+

dis. col., diseased colony with clinical symptoms.

HotStar*Taq* polymerase (Qiagen). The reaction conditions were as follows, after the initial activation step (15 min, 95 °C); 35 cycles at 94 °C for 1 min, 53 °C for 1 min and 72 °C for 2·5 min, followed by a final elongation step at 72 °C for 10 min. A 5 µl sample from the PCR was analysed on a 0·8 % agarose gel. DNA bands were stained with ethidium bromide and visualized by UV light.

PFGE. Bacterial cells were cultured on blood agar plates for 48–72 h at 34 °C. A loopful of bacteria from fresh culture was suspended in 5 ml pre-warmed (37 °C) LB broth and incubated with shaking (150 r.p.m.) at 37 °C for 16–18 h. Cultures were chilled on ice and the samples were kept cold in all further steps. Depending on cell density, 2·5 or 5 ml culture was centrifuged (4200 r.p.m., 10 min, 4 °C) and the pellet was washed once with 1 ml washing buffer [200 mM NaCl, 10 mM Tris/HCl (pH 7·2), 100 mM EDTA]. Bacterial cells were resuspended in 0·25 ml washing buffer, quickly warmed to 37 °C, mixed with an equal amount of 1·5 % InCert agarose (37 °C) and immediately pipetted into plug moulds. The plugs were allowed to solidify on ice and digested overnight with proteinase K solution (0·5 M EDTA pH 8·0, 1 % *N*-lauroylsarcosine, 1·5 mg proteinase K ml^{-1}) at 50 °C. The plugs were subsequently stored in 0·5 mM EDTA.

Before the analysis, the plugs were dialysed for 2–3 days against TEN buffer (10 mM Tris/HCl pH 7·4, 1 mM EDTA, 50 mM NaCl). The buffer was changed once during dialysis. DNA was digested with 10 U *Not*I for 16 h at 37 °C. PFGE was performed in the Bio-Rad CHEF DR III with 1 % pulsed-field certified agarose (Bio-Rad) in 0·5× Tris/borate/EDTA buffer at 14 °C. The switch time ramp was divided in two parts, 1·0–7·5 s for 17 h and 10·0–30·0 s for 5 h. The switch angle was 120° and the voltage was 6·0 V cm^{-1}. After PFGE, the gels were stained with ethidium bromide and photographed under UV transillumination. Macrorestriction profiles (MRPs) were analysed visually and with the GelCompar II program (Applied Maths). One fragment difference in MRPs was interpreted as being significant. The similarity of the MRPs was determined using the Dice coefficient and clustering was performed by the unweighted pair group method with arithmetic averages (UPGMA) with a 1·9–2·4 % tolerance.

16S rRNA gene sequencing. DNA was isolated from a loopful of bacterial cells using spin columns (NucleoSpin tissue kit; Macherey & Nagel) in 100 µl elution buffer according to the manufacturer's instructions. Extracted DNA was subjected to amplification in a final volume of 50 µl containing 0·5 µM of each oligonucleotide primer (GM3F, 5′-AGAGTTTGATCMTGGC-3′; GM4R, 5′-TACCTTGTTACGACTT-3′; Buchholz-Cleven *et al.*, 1997), 250 µM (each) dATP, dCTP, dGTP and dTTP (Amersham Biosciences), 1·5 mM $MgCl_2$ and 0·5 U DNA *Taq* polymerase (Qiagen). PCR was performed for 29 cycles of 45 s at 94 °C, 60 s at 50 °C and 90 s at 72 °C.

Double-stranded PCR products were sequenced directly with the primers used for PCR using the BigDye terminator cycle sequencing kit according to the manufacturer's protocol (Applied Biosystems). Additional primers were used to sequence upstream and downstream of the PCR product. Sequencing products were run on an ABI Prism 310 Genetic Analyzer. Sequences were edited with the OMIGA 2.0 program (Accelrys) and aligned using the CLUSTAL W algorithm (Thompson *et al.*, 1994). The 16S rRNA gene sequence data obtained from the isolates were compared with published sequences in the GenBank database using BLAST (Altschul *et al.*, 1990).

Electron microscopy. Spores of bacterial isolates were retrieved from cultures cultivated on MYPGP agar incubated at 36 °C in an atmosphere containing 5 % CO_2. Sporulating bacterial colonies were suspended in 4 % glutaraldehyde (v/v) in 0·067 M cacodylate buffer, pH 7·4, and kept refrigerated overnight. The spore suspension was then deposited on a polycarbonate filter (0·6 µm, Nuclepore) and dehydrated in a graded series of ethanol. The dehydration was completed in hexamethyldisilazane overnight. The dry filters were mounted on the sample holder by double-sided sticky tape, coated with Pt/Pd in a sputter coater and analysed in a scanning microscope at 5 kV (JSM 6320F; JEOL).

API. The strains were characterized biochemically using the commercially available API 50CHB and API 20E systems according to the manufacturer's instructions (bioMérieux).

API ZYM. All strains were analysed with the API ZYM test (bioMérieux). The test was performed according to the manufacturer's instructions with minor modifications. Briefly, *P. larvae* colonies from T-HCl-YGP agar were suspended in 3 ml saline to obtain a turbidity of 5–6 on the McFarland scale. Each well was filled with three to four drops of the suspension. The strip was incubated at 35 °C for 16–17 h.

Protein profiling. SDS-PAGE was performed at the CCUG. Polyacrylamide gel electrophoresis analysis of whole-cell proteins was performed as described by Pot *et al.* (1994). For densitometry analysis, normalization and interpretation of protein patterns, the GelCompar software package (Applied Maths), versions 3.0 and 4.2, was used. The similarity between all pairs of traces was expressed by the Pearson product–moment correlation coefficient, which was converted to percentage similarity for convenience.

Exposure bioassay. For rearing and infecting worker larvae, we used a modified protocol according to Peng *et al.* (1992). Briefly, larvae were reared in 24-well tissue culture plates. The wells were either filled with 300 µl larval diet (larval plate) or tightly lined with precut Kimwipes tissue (pupation plate). The grafted larvae were maintained in constant darkness in an incubator at 35 °C and 95 % relative humidity. Normal larval diet consisted of 3 % (w/v) fructose, 3 % (w/v) glucose and 66 % (v/v) royal jelly (purchased from a local bee keeper) in sterile double distilled water. For infection, the final spore concentration was adjusted using a working solution of 1×10^5 c.f.u. ml^{-1}. The infectious larval diet was fed to larvae for the first 24 h after grafting when the larvae were between 12 and

36 h of age. Thereafter, normal larval diet was used for feeding. Control larvae were fed with normal larval diet throughout the larval stages.

Three wells in a culture plate were filled with larval diet (normal or infectious). Ten larvae of the first larval instar were grafted into each well using a special grafting tool to avoid injuring the larvae. A total of thirty larvae per plate was treated as one replicate. One experiment consisted of four replicates: three infected groups and one control group. Larvae were infected with 300 c.f.u. ml^{-1} (ATCC 9545^T), 500 c.f.u. ml^{-1} (DSM 3615^T, LMG 6911^T) and 2000 c.f.u. ml^{-1} (LMG 16252, LMG 16247) to obtain a mortality of at least 50 %. All experiments were performed at least three times.

Each day, larvae were taken out of the incubator and examined under a stereo microscope for signs of respiration, injury, disease symptoms or other abnormalities. Larvae were classified as dead when respiration ceased, they lost their body elasticity or developed marked oedema and changed colour to greyish or brownish. The number of dead larvae in each well was recorded and surviving larvae were transferred to new wells freshly filled with food. On the third, fourth, fifth and sixth day, the growing larvae were placed in groups of five, three, two and one per well, respectively. After defecation, i.e. after clear uric acid crystals and light yellow excretions could be observed in the remaining diet, engorged larvae were transferred into pupation plates. To remove adherent food, larvae were gently rolled on Kimwipes tissue before transfer. Since the larvae often continued to defecate in the pupation plates, they were transferred into clean pupation plates until defecation stopped. Engorged larvae metamorphose through a prepupal and pupal stage before emerging as adults. At all stages of development, dead animals were classified as dead from AFB only when vegetative *P. larvae* could be cultivated from the larval remains. On no occasion was *P. larvae* cultivated from the remains of dead control animals. Mortality in the control group varied from 5 to 15 %. Experiments with a mortality exceeding 15 % in the control group were excluded, as were experiments where the 'natural' mortality (larval death but no growth of *P. larvae*) in the infected groups was higher than 15 %.

To obtain the time-course of infection, for each replicate ($n=30$) the number of dead larvae was recorded every day. From at least three independent experiments, the mean cumulative proportion of AFB-dead larvae per day $\pm$ standard deviation was calculated and plotted against time. Survivors were excluded from this calculation (Thomas & Elkinton, 2004). From the graph obtained, the LT_{100} (i.e. the time it took the pathogen to kill 100 % of the infected hosts) was determined.

To evaluate whether the larval remains develop into a typical AFB scale via a ropy stage with glue-like consistency or into a powdery scale, some of the dead larvae from the infected groups were put on glass slides in Petri dishes and remained unattended in the incubator. The consistency of the larval remains during the ropy stage was tested with a matchstick. Finally, the dried-down scale was evaluated before the spores were plated on agar plates to confirm the identity of the cultivated bacterial colonies.

RESULTS AND DISCUSSION

rep-PCR using ERIC primers

A recent study suggested that analysing *P. larvae* strains by rep-PCR using ERIC-primers results in two band patterns, each one specific for either *P. larvae larvae* or *P. larvae pulvifaciens*. In particular, a band of 970 bp was considered to be specific for *P. larvae larvae* (Alippi *et al.*, 2004). Genotyping of all *P. larvae* strains included in this study revealed a total of four different ERIC patterns, ERIC I–ERIC IV (Fig. 1). ERIC I and II showed the *P. larvae larvae*-specific band migrating at around 970 bp (Fig. 1, lanes 2–8), but differed in one prominent band migrating at around 2800 bp and in one minor band migrating at around 1200 bp. Field isolates from bee hives showing the classical symptoms of AFB fell into one of these two ERIC-groups. All orange-coloured *P. larvae larvae* strains belonged to the *P. larvae larvae*-specific ERIC pattern II, exhibiting an additional band migrating at 2800 bp (Fig. 1, lanes 6–8). The reference strains for *P. larvae larvae* and all field isolates not growing with pigmented colonies belonged to the *P. larvae larvae*-specific ERIC pattern I, not featuring the 2800 bp band (Fig. 1, lanes 2–5). The reference strains for *P. larvae pulvifaciens* revealed two more ERIC patterns (III, IV) resembling the *P. larvae larvae*-pattern. These patterns did not possess any band migrating at around 970 bp (Fig. 1, lanes 9–13). Strain LMG 16252 was the only representative of ERIC pattern III, characterized by a double band migrating between 1500 and 2000 bp (Fig. 1, lane 9) also present in ERIC patterns I and II. Strains ATCC 49843^T, LMG 16247, LMG 6911^T and DSM 3615^T belonged to group IV, characterized by having no bands migrating more slowly than 1250 bp (Fig. 1, lanes 10–13). Although Alippi *et al.* (2004) described only one ERIC pattern for *P. larvae larvae*, the existence of the ERIC I and ERIC II patterns is consistent with an earlier report describing two ERIC patterns among German field isolates which correlated with the two patterns obtained by the same method using MBO REP1 primers (Genersch & Otten, 2003). The two other ERIC patterns,

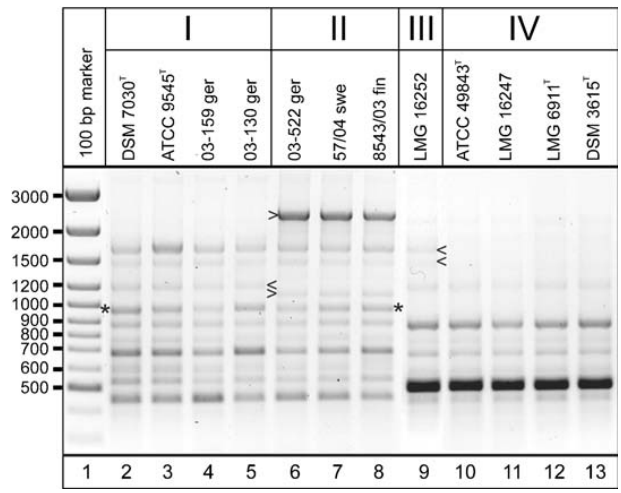

Fig. 1. Genotyping via rep-PCR using ERIC primers. Using the primer pair ERIC1R/ERIC2, field isolates and reference strains of *P. larvae larvae* (DSM 7030^T, ATCC 9545^T) as well as reference strains for *P. larvae pulvifaciens* (LMG 16252, ATCC 49843^T, LMG 16247, LMG 6911^T, DSM 3615^T) were analysed. The band migrating at 970 bp, considered specific for the subspecies *P. larvae larvae*, is indicated by a star. Bands important for the differentiation of the four ERIC genotypes are highlighted by arrowheads.

ERIC III and ERIC IV, obtained for the reference strains of *P. larvae pulvifaciens* lack the 970 bp band, suggesting that this feature could be indicative for *P. larvae pulvifaciens* strains.

PFGE

Applying PFGE, the gold standard for genotyping (Olive & Bean, 1999), revealed that the MRPs of *P. larvae* strains were quite similar (77 % overall similarity) and could be divided into two main clusters (Fig. 2). One cluster contained all isolates belonging to the *P. larvae* genotypes ERIC I (non-pigmented field isolates as well as *P. larvae larvae* type strain ATCC 9545^T), ERIC IV (*P. larvae pulvifaciens* reference strains LMG 16247 and ATCC 49843^T) and ERIC III (red-pigmented *P. larvae pulvifaciens* reference strain LMG 16252). The MRPs of the three *P. larvae pulvifaciens* reference strains were very similar, but not identical and they formed one group that differed in many ways from the profile of the typical *P. larvae larvae* strains belonging to the ERIC I genotype, which showed 85 % internal similarity. The other main cluster contained all *P. larvae larvae* field isolates belonging to the pigmented genotype ERIC II. All of these strains had many conserved regions and the MRPs were very similar (88 % similarity). This cluster could be divided into two groups differing in a band at 290 kb. All Finnish strains showed a band migrating at 290 kb that was not found in the German and Swedish strains. Since the three *P. larvae pulvifaciens* reference strains were more closely related to the typical *P. larvae larvae* strains, including the *P. larvae larvae* type strain ATCC 9545^T, than to the pigmented *P. larvae larvae* strains belonging to the ERIC II genotype, PFGE analysis did not support the classification of the *P. larvae* genotypes ERIC III and ERIC IV as a separate *P. larvae pulvifaciens* subspecies.

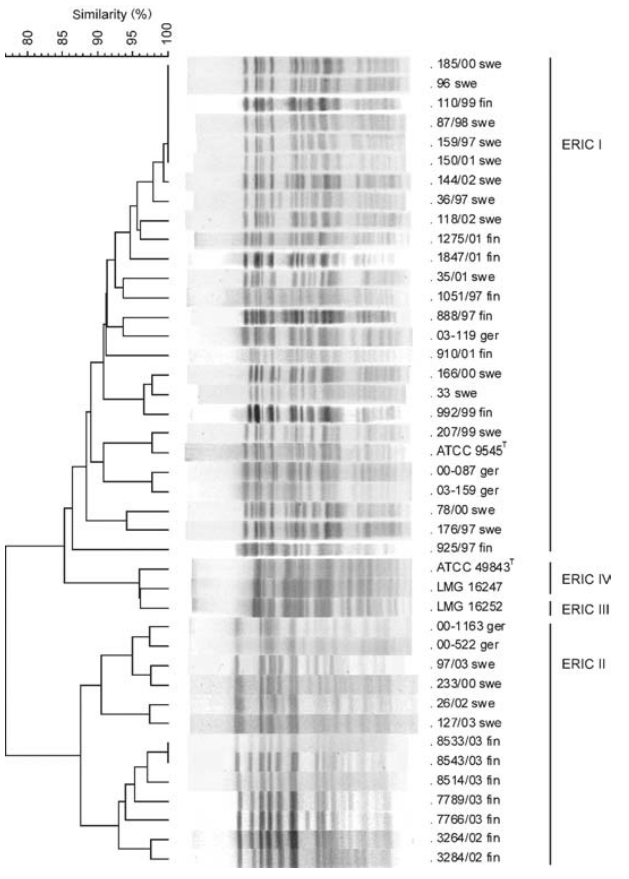

Fig. 2. Dendrogram based on the UPGMA clustering of normalized computer profiles of a PFGE analysis of *P. larvae* strains. Using PFGE, *Not*I MRPs of *P. larvae larvae* field isolates and reference strain ATCC 9545^T, as well as of *P. larvae pulvifaciens* reference strains ATCC 49843^T, LMG 16247 and LMG 16252, were separated. The attribution of the strains to the four ERIC genotypes is indicated.

Comparative analysis of 16S rRNA gene sequences

Comparative analysis of the almost-complete 16S rRNA gene sequences of *P. larvae larvae* field isolates 03-525 (DSM 16116), 00-1163, 02-130, 03-189 (GenBank accession numbers DQ079620, DQ079621, DQ079622, DQ079623, respectively) with sequences of *P. larvae larvae* and *P. larvae pulvifaciens* reference strains available through GenBank showed high similarity (> 99·5 %). Over the entire sequence range analysed (1516 bp), only six positions were polymorphic (Table 2). Field isolate 03-189 showed the most divergent 16S rRNA genotype, with four positions differing from the sequence of the *P. larvae larvae* type strain, DSM 7030^T (GenBank accession number AY530294). Nevertheless, it still had 99·7 % sequence similarity. Contradictory to the subspecies classification, the sequence of the type strain of *P. larvae pulvifaciens*, ATCC 49843^T (GenBank accession number AY030080), was more closely related to the sequence of the type strain of *P. larvae larvae*, DSM 7030^T, than to the sequence of 03-189, a field isolate of *P. larvae larvae*. This correlates well with a recent study demonstrating 100 % similarity between the 16S rRNA gene sequences of strains DSM 3615^T and DSM 7030^T (Kilwinski *et al.*, 2004). Therefore, an evaluation of the relationship between *P. larvae larvae* reference strains, *P. larvae larvae* field isolates and *P. larvae pulvifaciens* reference strains, based on the 16S rRNA gene sequence, strongly suggests that the isolates belong to one species which cannot be subdivided into two subspecies.

In summary, genotypic characterization of the studied isolates demonstrated no consistent differences in rep-PCR patterns, PFGE fingerprinting or DNA sequencing data that can be used to subclassify the *P. larvae* species.

Phenotypic characterization

In the literature, some phenotypic characteristics that discriminate *P. larvae pulvifaciens* and *P. larvae larvae* are described (Heyndrickx *et al.*, 1996), such as colony pigmentation, spore morphology, growth in nutrient broth and the metabolism of mannitol and salicin.

Table 2. Polymorphic positions within 16S rRNA gene sequences

Changes are indicated relative to the sequence of *P. larvae larvae* DSM 7030^T (AY530294). Pos., position; +, positive; −, negative; ND, not determined.

Strain	GenBank accession no.	Pos. 52 A→C	Pos. 53 A→deleted	Pos. 75 T→deleted	Pos. 737 A→G	Pos. 1022 T→C	Pos. 1465 C→G
DSM 3615^T	AY530295	−	−	−	−	−	−
ATCC 49843^T*	AY030080	+	+	+	−	−	ND
NRRL B-3555	AY030079	−	−	+	+	+	ND
03-525 (=DSM 16116)	DQ079620	−	−	−	−	+	−
00-1163	DQ079621	−	−	−	−	+	−
02-130	DQ079622	−	−	+	+	+	−
03-189	DQ079623	−	−	+	+	+	+

*The sequence of ATCC 49843^T starts at position 49 of the DSM 7030^T sequence; therefore, positions 52/53 relate to positions 4/5 of the ATCC 49843^T sequence. Hence, these polymorphisms might be the result of sequence errors that occur frequently near the beginning of a sequencing reaction.

Among the reference strains for *P. larvae pulvifaciens*, strain LMG 16252 (ERIC III) grew with orange–red colonies when cultivated on sheep blood agar plates. This strain was the only one that also showed strong haemolysis on sheep blood agar plates. The other reference strains (ERIC IV) revealed a colony morphology with a whitish to greyish, somewhat transparent and slightly glistening appearance, as did the two type strains for *P. larvae larvae*, DSM 7030^T and ATCC 9545^T (ERIC I). The field isolates of *P. larvae larvae* showed both kinds of colony morphology, whitish (ERIC I) and orange-pigmented (ERIC II) (see Supplementary Fig. S1 in IJSEM Online and Table 3).

Electron microscopy revealed that spores of the *P. larvae larvae* type strain, ATCC 9545^T (ERIC I), had a smooth surface while spores of an orange-pigmented *P. larvae larvae* field isolate, 233/00 swe (ERIC II), had a convoluted surface resembling the surface of a brain. In contrast, the spore surface of the *P. larvae pulvifaciens* type strain DSM 3615^T (ERIC IV) was characterized by ridges running longitudinally along the slightly rod-shaped spores. In addition to these ridges, the surface of spores of the pigmented *P. larvae pulvifaciens* reference strain LMG 16252 (ERIC III) also showed smaller ridges lying mostly perpendicular to and between the longer ridges (see Supplementary Fig. S2 in IJSEM Online for scanning electron microscopy images of spores of all four genotypes). These results are consistent with earlier reports. While the surface of *P. larvae larvae* spores was described as smooth (Alippi, 1991), the spores of *P. larvae pulvifaciens* showed characteristic ridges on the surface (Ludvik *et al.*, 1993). The convoluted surface of *P. larvae larvae* isolates belonging to the pigmented variant has not been described previously. Although *P. larvae pulvifaciens* has been described as having ridges running longitudinally along the rod-shaped spores, in contrast to the smooth surface of *P. larvae larvae* spores (Ludvik *et al.*, 1993), this difference can no longer be used to distinguish between the two *P. larvae* subspecies since one of the type

Table 3. Phenotypic characteristics of *P. larvae* ERIC genotypes

All genotypes have ellipsoidal spores and are pathogenic for honey bees. Diseased larvae show clinical symptoms of AFB. Larval remains have a glue-like consistency and develop into a hard scale.

Characteristic	ERIC I	ERIC II	ERIC III	ERIC IV
Pigmented colonies	−	+	+	−
Haemolysis	−	−	+	−
Spore surface	Smooth	Convoluted	With ridges	With ridges
Subculturing in nutrient broth	−	+	+	+
Fermentation of mannitol	−	+	+	+
Fermentation of salicin	+	−	−	−
Alkaline phosphatase	+	−	+	+
Acid phosphatase	+	−	+	+
LT_{100} (days)	~ 12	~ 7*	~ 7	~ 7

*Data from Genersch *et al.*, 2005.

strains for *P. larvae pulvifaciens* (DSM 3615^T) containing such ridges has unambiguously been characterized as *P. larvae larvae* (Kilwinski *et al.*, 2004).

According to Heyndrickx *et al.* (1996), *P. larvae larvae* is unable to grow in nutrient broth on repeated subculture while *P. larvae pulvifaciens* is able to do so. Our data revealed that growth behaviour in nutrient broth on repeated subculture depends on genotype (Table 3). Only *P. larvae larvae* of the ERIC I genotype was unable to grow under these conditions, while the pigmented *P. larvae larvae* of the ERIC II genotype and the reference strains for *P. larvae pulvifaciens* grew well on repeated subculture in nutrient broth. All genotypes could be subcultured on plates repeatedly if colonies were resuspended in nutrient broth before streaking them onto new agar plates.

P. larvae larvae is described as metabolizing salicin (may be weak), but not mannitol, whereas *P. larvae pulvifaciens* is described as being able to use mannitol but not salicin (Heyndrickx *et al.*, 1996). Analysing all strains using the API 50CHB and API 20E system indeed revealed significant differences in the metabolism of these two carbon sources. But, in contrast to the results of Heyndrickx *et al.* (1996), in our data, subspecies allocation did not correlate with metabolic phenotype. Only the non-pigmented *P. larvae larvae* strains (ERIC I) were able to use salicin, but not mannitol (Table 3). All other strains showed the opposite characteristics and included not only the *P. larvae pulvifaciens* reference strains, but also the pigmented *P. larvae larvae* genotype ERIC II, suggesting that the metabolism of mannitol and salicin was genotype-specific rather than specific for the subspecies *P. larvae larvae* and *P. larvae pulvifaciens*. Further biochemical analysis using the API ZYM technique revealed differences in the activity of acid and alkaline phosphatases (Table 3). Using this method, the *P. larvae pulvifaciens* reference strains clustered together with the ERIC I-genotype of *P. larvae larvae*. They showed a positive reaction for acid- and alkaline-phosphatases, while the pigmented *P. larvae larvae* genotype ERIC II was negative for alkaline phosphatase and showed only a weak reaction for acid phosphatase. These results confirm and expand earlier reports of genotype-specific differences in the metabolism and biochemistry of *P. larvae larvae* (Neuendorf *et al.*, 2004), but they do not support the classification of two subspecies within the species *P. larvae*.

SDS-PAGE

Analysing the protein profiles of the *P. larvae* strains revealed two clusters (Fig. 3). One cluster comprised all but one of the pigmented *P. larvae larvae* field isolates (ERIC II) and the *P. larvae pulvifaciens* reference strains LMG 16252 (ERIC III), CCUG 7427 and LMG 16247 (both ERIC IV). The other cluster included all the non-pigmented field isolates and the reference strain CCUG 28515^T of *P. larvae*

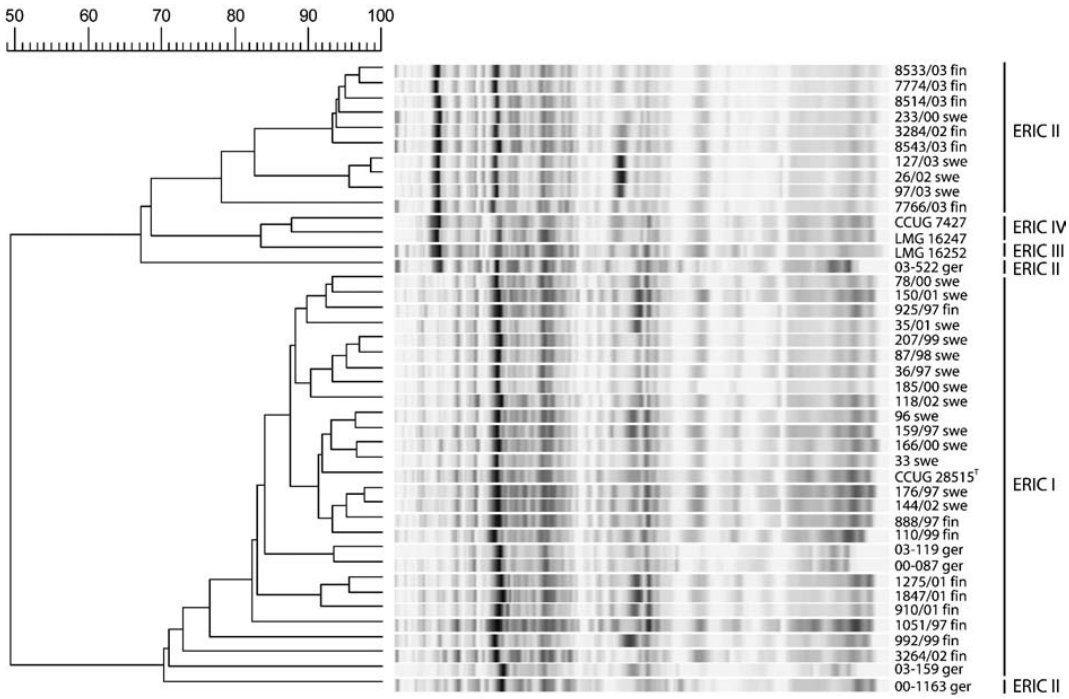

Fig. 3. Dendrogram based on the UPGMA clustering of normalized computer profiles of protein patterns (SDS-PAGE) of *P. larvae* strains. Field isolates of *P. larvae larvae* and reference strain CCUG 28515^T, as well as *P. larvae pulvifaciens* reference strains CCUG 7427, LMG 16247 and LMG 16252, were analysed by SDS-PAGE. Attribution of the strains to the four different ERIC genotypes is indicated. Bar, % similarity.

larvae (ERIC I), as well as one pigmented field isolate. The two clusters exhibited only 49 % similarity; within each cluster the similarity was 68 %. Such a low clustering level within the species *P. larvae* has already been described by Heyndrickx *et al.* (1996). In this study, clustering above 82 % could only be achieved by leaving out all bands with molecular masses of more than $1{\cdot}3 \times 10^5$. However, *P. larvae pulvifaciens* reference strains clustered separately from *P. larvae larvae* reference strains, supporting the notion of two different infraspecific groups (Heyndrickx *et al.*, 1996). Our data reveal that the *P. larvae pulvifaciens* reference strains and some, but not all, of the pigmented *P. larvae larvae* genotype form one cluster, clearly differing from the other cluster. This confirms infraspecific differences, but does not support the classification of *P. larvae* into two separate subspecies based on current subspecies descriptions (Heyndrickx *et al.*, 1996).

Exposure bioassays

A strong argument for the classification of *P. larvae* into two subspecies has been the different pathologies of *P. larvae larvae* and *P. larvae pulvifaciens* (Heyndrickx *et al.*, 1996). *P. larvae larvae* is a highly virulent pathogen of honey bee larvae causing AFB, whereas *P. larvae pulvifaciens* is considered as an almost avirulent pathogen causing the extremely rare powdery scale disease of honey bee larvae (Katznelson, 1950; Heyndrickx *et al.*, 1996). Exposure bioassays revealed that all representatives of the species *P. larvae*, including all reference strains for *P. larvae pulvifaciens*, were pathogenic to honey bee larvae (Table 3). In contrast to the situation described so far from field observations, larvae experimentally infected with *P. larvae pulvifaciens* never developed into a powdery scale, but the larval remains formed a glue-like colloid which dried down to a hard scale as described for AFB (Table 3). In addition, when the LT_{100} for the different strains were compared, the *P. larvae pulvifaciens* reference strains proved to be even more virulent than the *P. larvae larvae* type strain ATCC 9545^T. While it took the *P. larvae pulvifaciens* reference strains as little as 7 days to kill all infected larvae, it took strain ATCC 9545^T around 12 days to kill 100 % of the infected hosts (Fig. 4). This fast-killing phenotype is not restricted to the *P. larvae pulvifaciens* reference strains as it can also be observed for isolates belonging to the *P. larvae larvae* ERIC II group (Genersch *et al.*, 2005). Therefore, different pathologies for *P. larvae larvae* and *P. larvae pulvifaciens* cannot be confirmed by exposure bioassays. At present, we have no explanation for the field observations resulting in the description of a powdery scale disease (Katznelson, 1950).

In summary, phenotypic characterization of the studied isolates demonstrates no consistent differences in spore morphology, culture characteristics, utilization of carbon sources, enzyme activity or protein profiles that can be used to differentiate between subspecies of *P. larvae*.

The data presented here support the conclusion that the reference and type strains for the subspecies *P. larvae*

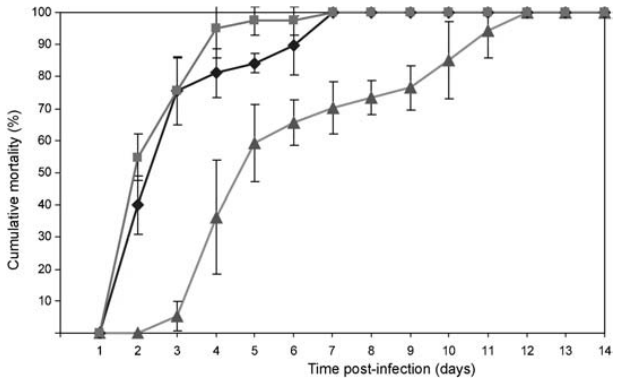

Fig. 4. Course of infection and determination of LT_{100}. *P. larvae larvae* and *P. larvae pulvifaciens* reference strains were tested for pathogenicity and virulence in exposure bioassays. To determine the LT_{100}, mortality was monitored every day and the cumulative mortality was calculated and expressed as percentage of all infected hosts, i.e. all larvae and pupae that died from AFB. Representative results obtained for three genotypes are shown. ▲, ERIC I (ATCC 9545^T); ◆, ERIC III (LMG 16252); ■, ERIC IV (DSM 3615^T).

pulvifaciens represent two genotypes within the species *P. larvae*. In a polyphasic analysis comprising morphological and biochemical tests, genomic fingerprints and exposure bioassays, these genotypes always clustered together with at least one of the two other genotypes of *P. larvae*, which were unambiguously identified as *P. larvae larvae* via a subspecies-specific PCR method (Alippi *et al.*, 2004).

As compelling evidence, based on a polyphasic approach, shows that the two subspecies represent variants of one pathogen that differs in virulence, it is proposed to that both the former subspecies should be reclassified as one species, *P. larvae*. No differences between *P. larvae pulvifaciens* and *P. larvae larvae* that justify the existence of two subspecies within the species *P. larvae* could be demonstrated. In particular, no differences in pathology could be demonstrated in exposure bioassays. Thus, the OIE definitions of the causative agent of AFB in honey bees need to be amended accordingly. An emended description of *P. larvae* is given.

Emended description of *Paenibacillus larvae*

The morphological, chemotaxonomic and general characters of *Paenibacillus larvae* are as already described for this species (Heyndrickx *et al.*, 1996). All representatives of this species are pathogenic to honey bee larvae, but differ in virulence. Decaying larvae will develop a ropy thread and a non-powdery scale. Depending on the LT_{100}, infected or dead larvae will be removed by the nurse bees before entering the pupation stage or left decaying in the capped cell. Growth in nutrient broth on repeated subculture depends on genotype. Strains will produce non-pigmented or yellow–orange- to red-pigmented colonies when cultivated

on Columbia blood agar or MYPGP agar, depending on genotype. Different isolates may be catalase-negative or weak-delayed positive, depending on age and genotype. The type strain of the former subspecies *P. larvae larvae*, ATCC 9545^T (=DSM 7030^T=CCUG 28515^T), could now serve as the reference strain for *P. larvae* genotype ERIC I. Two field isolates of genotype ERIC II, 03-522ger and 03-525ger, have been deposited as DSM 16115 and DSM 16116, respectively. All Swedish and Finnish field isolates of genotype ERIC II, as well as two German isolates of genotype ERIC II, 00-1163ger and 03-522ger, have been deposited at CCUG. A table detailing all strains deposited at CCUG with the respective accession numbers is available as Supplementary Table S1 in IJSEM Online). Only one reference strain of genotype ERIC III (Gilliam 2=NRRL NRS-1684=LMG 16252) is available, originating from a single isolation (Gilliam & Dunham, 1978). Several type and reference strains for the former subspecies *P. larvae pulvifaciens*, ATCC 49843^T (=DSM 3615^T=LMG 6911^T), LMG 16247 and CCUG 7427, can now serve as reference strains for the *P. larvae* genotype ERIC IV.

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