Available online at www.sciencedirect.com
SCIENCE DIRECT®
Veterinary Microbiology 104 (2004) 31–42
veterinary microbiology
www.elsevier.com/locate/vetmic

Proposal to reclassify *Paenibacillus larvae* subsp. *pulvifaciens* DSM 3615 (ATCC 49843) as *Paenibacillus larvae* subsp. *larvae*. Results of a comparative biochemical and genetic study

Jochen Kilwinski[a], Martin Peters[a], Ainura Ashiralieva[b], Elke Genersch[b,*]

[a]*Staatliches Veterinäruntersuchungsamt Arnsberg, Zur Taubeneiche 10-12, 59821 Arnsberg, Germany*
[b]*Länderinstitut für Bienenkunde, Friedrich-Engels-Str. 32, 16540 Hohen Neuendorf, Germany*

Received 6 February 2004; accepted 4 August 2004

Abstract

The bacterial pathogen *Paenibacillus larvae* subsp. *larvae* (*P. l. larvae*), is the etiological agent of American foulbrood, an extremely contagious and disastrous disease of honeybee brood. In case of American foulbrood the destruction of infected colonies is often considered the only workable control measure. Therefore, the ability to diagnose this disease properly is important to prevent unnecessary economic loss to beekeepers. The development of suitable methods for the early and reliable detection of *P. l. larvae* is hampered by the fact that the two subspecies of *Paenibacillus larvae*, *P. l. larvae* and *Paenibacillus larvae* subsp. *pulvifaciens* (*P. l. pulvifaciens*), seem to be indistinguishable by cultural characteristics as well as by PCR protocols. Here we present an extensive analysis of several *P. larvae* reference strains. We employed conventional culture techniques, morphological and biochemical identification, PCR-based methods and sequencing of the 16S rDNA. We found indeed that *P. l. pulvifaciens* strain DSM 3615 is indistinguishable from *P. l. larvae* (DSM 7030). We did not face any problems to discriminate between *P. l. larvae* and *P. l. pulvifaciens* strains DSM 8442 and DSM 8443. Therefore, classification of DSM 3615 as type strain of *P. l. pulvifaciens* seems not to be justified. We propose to reclassify this strain as *P. l. larvae*. Former problems in differentiating the two subspecies might have arisen from this misclassification. PCR-based methods as well as appropriate biochemical identification systems provide a reliable means for the discrimination between the two subspecies *P. l. larvae* and *P. l. pulvifaciens*.

Keywords: American foulbrood; *Paenibacillus larvae larvae*; *Paenibacillus larvae pulvifaciens*; 16S rRNA gene sequencing; Biochemical fingerprinting

* Corresponding author. Tel.: +49 3303 293833; fax: +49 3303 293840.
E-mail address: elke.genersch@rz.hu-berlin.de (E. Genersch).

0378-1135/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2004.08.001

1. Introduction

The Gram-positive, spore-forming bacterium *Paenibacillus larvae* subsp. *larvae* (*P. l. larvae*), is the etiological agent of American foulbrood (AFB), the most serious bacterial disease of honeybee larvae. AFB is an extremely contagious disease, which readily spreads within an apiary by just moving a comb from one hive to the other as well as between apiaries by e.g. robbery or drifting bees. AFB has spread around the world and in many countries AFB is a notifiable disease and, therefore, control measures are regulated by corresponding laws.

Paenibacillus larvae subsp. *pulvifaciens* (*P. l. pulvifaciens*) is a close relative to *P. l. larvae*. It is also considered a bee pathogen, most likely causing the rare Powdery scale disease of honeybee brood (Katznelson, 1950; Bailey and Ball, 1991).

The diagnosis of an outbreak of AFB is based on clinical symptoms and on the isolation and identification of *P. l. larvae* from diseased brood. Once an AFB outbreak is officially stated most authorities consider destruction of infected colonies by burning the only workable control measure. All spore contaminated material which cannot be disinfected by e.g. sodium hydroxide or flaming must also be destroyed by burning. Hence, AFB is causing great economical loss to beekeepers all over the world.

The oval-shaped spores represent the infectious stage of *P. l. larvae*. AFB is transmitted through spore-containing honey being fed to newly hatched larvae. Therefore, early diagnosis of infected although not necessarily already clinically diseased bee colonies is possible by detection of *P. l. larvae* spores in honey samples. Prophylactic measures, like AFB monitoring programs, are mostly based on such examinations. If spores are detected in the absence of clinical symptoms in most countries an outbreak of AFB cannot be officially declared. This opens the possibility to the beekeeper of curing the infected hives by e.g. artificial swarms, thus preventing the total loss of bees and material.

Since the consequences of a *P. l. larvae*-positive result are serious it is absolutely necessary to be able to unambiguously identify *P. l. larvae* and to discriminate between *P. l. larvae* and close relatives like *P. l. pulvifaciens*.

Mostly, diagnosis of *P. l. larvae* is based on conventional culture methods followed by further analysis of suspect colonies, which showed cultural characteristics indicative for *P. l. larvae*. To be diagnosed as *P. l. larvae* a colony must meet several additional criteria, like being catalase-negative and forming giant whips upon sporulation.

Recently, different PCR-based methods for the identification of *P. l. larvae* were published (Govan et al., 1999; Dobbelaere et al., 2001b; Lauro et al., 2003). Although, PCR is a very powerful tool in the exact identification of and discrimination between pathogens it hitherto seemed to be impossible or at least complicated to distinguish between *P. l. larvae* and *P. l. pulvifaciens* by PCR methods. Originally, the PCR detection method was developed for the identification of the species *P. larvae* without any subspecies differentiation (Govan et al., 1999). In ensuing studies (Dobbelaere et al., 2001b; Lauro et al., 2003) the possibility to discriminate between the two subspecies *P. l. larvae* and *P. l. pulvifaciens* was analyzed and it was stated that PCR-based methods are not suitable for this purpose. To evaluate the possibilities to discriminate between these two subspecies of *P. larvae* using molecular methods we extensively analyzed *P. l. pulvifaciens* reference strains DSM 3615, 8842 and 8443 and *P. l. larvae* reference strain DSM 7030. We employed conventional culture as well as several molecular methods, like diagnostic PCR, biochemical fingerprinting and sequencing of 16S rDNA.

2. Materials and methods

2.1. Bacterial isolates

Reference strains for *P. l. larvae* and *P. l. pulvifaciens* (DSM 7030 and DSM 3615, 8442, 8443, respectively) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. *P. l. larvae* DSM 7030 is identical to reference strain ATCC 9545. *P. l. pulvifaciens* DSM 3615, DSM 8442 and DSM 8443 are identical to ATCC 49843, NRRL NRS1683 and NRRL NRS1684, respectively (see also Table 3).

2.2. Bacterial culture

P. larvae reference strains DSM 7030, DSM 3615, DSM 8442 and DSM 8443 were cultured on Columbia

sheep blood agar plates exactly as given by the DSMZ. For further analysis, reference strains were stored as bacterial suspensions in 25% glycerol in brain heart infusion (BHI) broth at −70 °C.

Cultivation of *P. larvae* from these bacterial suspensions was performed by thawing the suspension at 4 °C prior to streaking them onto Columbia sheep blood agar plates using a sterile wooden stick. Three plates were prepared from each sample. Plates were incubated at 37 °C and evaluated for bacterial growth after 3 and 6 days. After 6 days the identity of the colonies was verified again by catalase- and Plagemann-tests as well as by specific PCR-detection prior to subsequent detailed analysis.

2.3. Catalase- and Plagemann-tests

All *P. larvae*-cultures were analyzed by catalase- and Plagemann-tests. For the catalase test, part of a colony of each bacterial culture was transferred to a microscopic slide using a sterile wooden stick and mixed with a drop of 3% H_2O_2. Production of air bubbles is indicative for catalase activity, whereas no air bubbles indicate missing catalase activity. *P. larvae* is described as catalase negative or weak, delayed positive (Heyndrickx et al., 1996). For the Plagemann-test (Plagemann, 1985), the liquid part of Columbia sheep blood agar slants was inoculated with one bacterial colony taken off the Columbia sheep blood agar plates. The tube was air tight sealed with Parafilm and incubated at 37 °C for 10 days. Subsequently, the liquid part was analyzed for the presence of spores and giant whips by phase contrast microscopy. For the subspecies *P. l. larvae* giant whips occurring upon sporulation are characteristic (Ritter, 1996; Hansen and Brodsgaard, 1999). Formation of giant whips upon sporulation was also observed for all three *P. l. pulvifaciens* strains.

2.4. P. l. larvae specific PCR-reaction

For PCR identification of bacterial colonies grown on agar plates, part of the colony in question was resuspended in 50 μl bidistilled water and subsequently incubated at 90 °C for 15 min. Probes were centrifuged at 5000 × *g* for 10 min. The supernatant containing the DNA was transferred to a new tube and directly used for PCR analysis. PCR analysis was based on 16S rDNA sequences of *P. l. larvae* (accession numbers AY030079 for strain NRRL B-3555 and X60619 for strain ATCC9545) and on the partial sequence of the gene for a 35 kD-metalloprotease from *P. l. larvae* (accession number AF111421). Primer sequences were designed using MacVector 6.5 software and compared with published sequences in the GenBank databases using BLAST (Altschul et al., 1990):

Pll-16S E1: 5′- GCAAGTCGAGCGGACCTTGTG-3′
Pll-16S E2: 5′-AAACCGGTCAGAGGGATGTCA-AG-3′
Pll-16S F6: 5′-GCACTGGAAACTGGGAGACTTG-3′
Pll-16S B11: 5′-CGGCTTTTGAGGATTGGCTC-3′
Pll-MP F3: 5′-CGGGCAGCAAATCGTATTCAG-3′
Pll-MP B1: 5′-CCATAAAGTGTTGGGTCCTCTAA-GG-3′

PCR reactions were carried out as previously described (Neuendorf et al., 2004). About 5 μl of the PCR reactions were analyzed on a 0.8% agarose gel. The DNA bands were stained with ethidium bromide and visualized by UV light. Specific amplicons generated with Pll-16S P1/P2, Pll-16S F6/B11 and Pll-MP F3/B1 were 965 bp, 665 bp and 273 bp in length, respectively. Specificity of the amplicons was verified by sequencing.

2.5. Biochemical fingerprinting (api- and BIOLOG-system)

The metabolic pattern of the bacterial strains was tested with the api- (Biomérieux, France) and with the BIOLOG™-system (obtained through Oxoid, Germany).

The api 50CH-system allows the study of the carbohydrate metabolism of bacteria and consists of 50 microtubes. The first tube contains no substrate and is used as the negative control. The remaining 49 tubes contain a defined amount of dehydrated carbohydrate substrate. Fermentation is shown by a colour change in the tube due to acid production. For typing of the *Paenibacilli* strains the api 50CHB/E medium was used. The system enables the biochemical identification and typing of bacilli and related genera, but the identification database does not include *P. l. larvae*

and *P. l. pulvifaciens*. For bacilli the additional use of an api 20E (bioMérieux, France), which analyzes the ability of a bacterium to perform a set of specific biochemical reactions, is recommended and was carried out. The tests were performed according to the manufacturer's instructions. The bacterial strains were grown on Columbia sheep blood agar and the inoculum was checked with a densitometer to correspond a turbidity of McFarland 2. The strips were read after incubation for 24 and 48 h at 37 °C.

The BIOLOG™-system is a 95 carbon source test, where the ability of a bacterial isolate to metabolize these carbon sources is used for identification purposes. Cultivation and preparing of *P. larvae* isolates for metabolic analysis using the BIOLOG™-system was performed according to the manufacturer's instructions for spore-forming, Gram-positive rods, with minor modifications to meet the growth requirements of *P. l. larvae* (Neuendorf et al., 2004). Briefly, single pure colonies of *P. larvae* were subcultured on BUG-M-T agar plates (bacterial universal growth agar supplemented with 0.25% maltose and swapped with thioglycolate) with one colony per plate. For the analysis of one isolate, eight pure colonies of this isolate were subcultured on eight BUG-M-T plates. Growth of bacteria was allowed at 37 °C for 48 h since *P. l. larvae* is a slowly-growing bacterium. Subsequently, the inoculum was prepared and turbidity was adjusted to match the turbidity standard (Oxoid GmbH, Germany) at 28% $T \pm 2\%$ (OD: 0.55). A GP2 MicroPlate™ (Oxoid GmbH, Germany) was inoculated with 150 μl bacterial suspension per well and incubated at 37 °C for 24 h. Metabolic activity was determined by reading the plates (endpoint reading method) in a microplate reader (EL800, BIO-TEK Instruments) with a primary wavelength of 590 nm and a reference wavelength of 750 nm. Positive wells were then entered manually into the Biolog MicroLog1 (release 4.20) software. Interpretation of the results and identification of the bacterial strain in question was performed automatically by the software. If the obtained profile matches one of the standard profiles present in the data bank the system's software is able to identify the bacterium and to provide information on the probability of this identification. The identification database does include *P. l. larvae* but does not include *P. l. pulvifaciens*. All strains were analyzed at least in triplicate.

2.6. 16S rDNA sequencing

DNA was isolated from a loopful of bacterial cells using spin columns (NucleoSpin Tissue Kit, Macherey & Nagel, Düren, Germany) in 100 μl of 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′AGAGTTTGATC-AGAGTTTGATCMTGGC-3′; GM4R: 5′TACCTTG-TACCTTGTTACGACTT-3′, Buchholz-Cleven et al., 1997), 250 μM (each) dATP, dCTP, dGTP and dTTP (Amersham Biosciences, Freiburg, Germany), 1.5 mM $MgCl_2$ and 0.5 U of DNA *Taq* polymerase (Qiagen, Hilden, Germany). 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 protocol of the manufacturer (Applied Biosystems, Foster City, USA). 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 (Accelrys, Cambridge, UK) program and aligned by using the Clustal W algorithm (Thompson et al., 1994). The 16S rDNA sequence data obtained from the isolates was compared with published sequences in the GenBank databases using BLAST (Altschul et al., 1990).

3. Results

3.1. PCR-identification of P. larvae

PCR-based methods are a state-of-the-art means in the diagnosis of many pathogens. In order to establish a PCR protocol for the definite identification of *P. l. larvae* we used two primer pairs amplifying different regions from the 16S rDNA as well as one primer pair amplifying a 273 bp-fragment from the gene for a *P. l. larvae*-specific metalloprotease (Table 1). We tested these primers with the *P. l. larvae* reference strain DSM 7030 and with three different reference strains of *P. l. pulvifaciens*, DSM 3615, DSM 8442 and DSM 8443. All three primer pairs generated specific

Table 1
Sequences for the metalloprotease gene: Comparison of *P. l. larvae* PCR primer sequences to sequences of other species

P.l. larvae primer and species	Sequence	% Identity to *P.l. larvae* sequence
MP3 (forward)	5′-CGGGCAGCAAATCGTATTCAG-3′	100
P. thuringiensis	5′-CGGatcaCAgATgGTATaCgG-3′	62
P. caldolyticus	5′-CGGttcGCAAATgGTgTaCgG-3′	67
P. amyloliquefaciens	5′-CGGcgAcCAAATgaTtTaCgG-3′	62
P. polymyxa	5′-CGGctccCAAATgactTatgG-3′	48
P. brevis	5′-CGGcagcCAgATgGTtTatgG-3′	52
B. cereus	5′-CGGatcaCAgATgGTATaCgG-3′	62
B. megaterium	5′-tGGctctCAAATgGTATaCgG-3′	62
MP1 (reverse)	5′-CCTTAGAGGACCCAACACTTTATGG-3′	100
P. thuringiensis	5′-CtaTgagtGATCCtAcgaagTATGG-3′	52
P. caldolyticus	5′-CgaTgtccGACCCggCgaaaTAcGG-3′	48
P. amyloliquefaciens	5′-gCTTAtccaAtCCgACAaaaTAcGG-3′	56
P. polymyxa	5′-CaaTgtccaAtCCtACtCTgTAcGa-3′	48
P. brevis	5′-CCcTgcAGGACCCggCAgcgTATGG-3′	68
B. cereus	5′-CtaTgagtGAtCCtACgaagTATGG-3′	52
B. megaterium	5′-CaaTgagcaACCCAgCgaaaTATGG-3′	52

The nucleotide sequences of *P. l. larvae* PCR primers and corresponding regions of the nucleotide sequences of the neutral protease genes of seven closely related *Paenibacilli* and *Bacilli* species were compared. Accession numbers are as follows: *P. l. larvae*, AF111421; *P. thuringiensis*, L77763; *P. caldolyticus*, M63575; *P. amyloliquefaciens*, K02497; *P. polymyxa*, D00861; *P. brevis*, X61286; *B. cereus*, M83910; *B. megaterium*, X75070. Nucleotide sequence identity with *P. l. larvae* is indicated by capital letters.

amplicons when used with *P. larvae* strains DSM 7030 and DSM 3615. In contrast, no amplification products could be observed with strains DSM 8442 and DSM 8443 (Fig. 1).

3.2. Biochemical fingerprinting of *P. larvae* strains

We applied the api 50CH-system to metabolically characterize the *P. larvae* reference strains DSM 7030, DSM 3615, DSM 8442 and DSM 8443. In Table 2A, the three different metabolic profiles obtained are depicted: The carbohydrate metabolic pattern of *P. l. pulvifaciens* strains DSM 8442 and DSM 8443 was identical with both strains reacting positive for glycerol, ribose, glucose, fructose, mannose, *N*-acetyl-glucosamine, arbutin, esculin, salicin, maltose and trehalose. The carbohydrate utilization of *P. l. larvae* DSM 7030 and *P. larvae* DSM 3615 were similar to each other but differed in the ability to use glycerol, salicin and mannitol. While *P. l. larvae* strain DSM 7030 is able to metabolize glycerol and salicin, *P. larvae* strain 3615 does not use these two carbohydrates. Mannitol is solely accepted as metabolic source by *P. larvae* DSM 3615. All other strains reacted negative for this carbohydrate. Differences between the profiles of the strains DSM 8442/DSM 8443 and the strains DSM 3615/DSM 7030 consist in the metabolism of fructose, arbutin, esculin and maltose which are not metabolized by the latter two strains.

Using the api 20E system, the only positive reaction was observed for gelatinolytic activity. *P. l. pulvifaciens* strains DSM 8442 and DSM 8443 as well as *P. larvae* strain DSM 3615 were able to degrade gelatine. *P. l. larvae* DSM 7030 showed only weak gelatinolytic activity (Table 2B).

For a more sophisticated metabolic analysis we used the BIOLOG system, which allows the identification of different *Paenibacillus* species including *P. l. larvae*. In Table 2C the metabolic profiles of all four *P. larvae* strains as compared to the BIOLOG standard profile are given. All four strains revealed different metabolic patterns. Based on the similarities they could be grouped into two subgroups with *P. l. larvae* DSM 7030 and *P. larvae* DSM 3615 comprising one group resembling the BIOLOG standard profile for *P. l. larvae* and *P. l. pulvifaciens* DSM 8442 and DSM 8443 comprising the other subgroup not related to the *P. l. larvae* BIOLOG standard. In accordance, the BIOLOG system identified both, *P. l. larvae* DSM 7030 and *P. larvae* DSM 3615, as

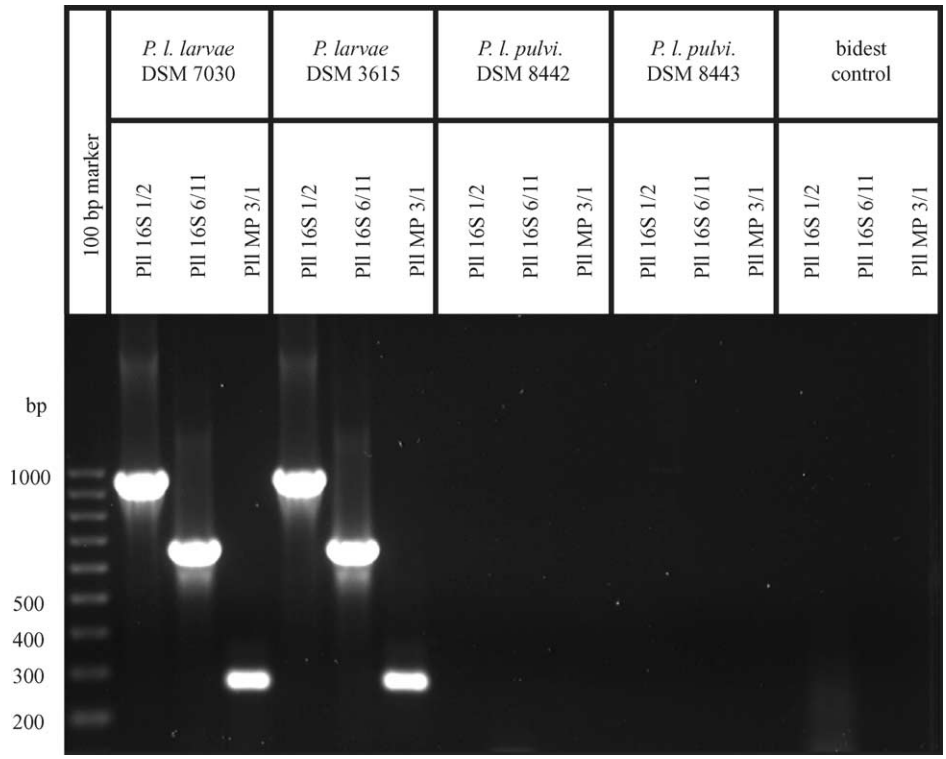

Fig. 1. PCR-identification of *P. larvae*. PCR-analysis of different *P. larvae* strains using primer pairs Pll-16S E1/E2 (Pll 16S 1/2) and Pll-16S F6/B11 (Pll 16S 6/11) for amplification of 16S rDNA fragments and primer pair Pll-MP F3/B1 (Pll MP 3/1) for amplification of a metalloproteinase gene segment. PCR products were analyzed on a 0.8% agarose gel in the presence of appropriate molecular size markers.

P. l. larvae with a probability ranging between 96% and 99%; *P. l. pulvifaciens* strains DSM 8442 and DSM 8443 obtained no identification. The main differences between the two groups lay in the metabolism of adenosine, 2′-deoxy-adenosine, inosine, thymidine, uridine, adenosine-5′-monophosphate, thymidin-5′-monophosphate and uridine-5′-monophosphate which could be metabolized by *P. l. larvae* DSM 7030 and *P. larvae* DSM 3615 but not by *P. l. pulvifaciens* DSM 8442 and DSM 8443 and within dextrin, Tween 80, L-arabinose, arbutin, D-fructose, lactulose, maltose, maltotriose, a-methyl-D-mannose, D-psicose, D-ribose, salicin, D-tagatose, D-xylose, acetic acid, β-hydroxy-butyric acid, γ-hydroxy-butyric acid, α-ketoglutaric acid, α-ketovaleric acid, *N*-acetyl-L-glutamic acid, L-alaninamid, L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, D-fructose-6-phosphate, D-glucose-6-phosphate and D,L-α-glycerol-phosphate which could not be metabolized by the *P. l. larvae*-subgroup but rather by the *P. l. pulvifaciens*-subgroup.

3.3. 16S rDNA sequencing

The sequences of the partial 16S rRNA gene fragment of *P. l. larvae* (strain DSM 7030), *P. l. pulvifaciens* (strains DSM 8442 and DSM 8443) and DSM 3615 have been deposited under Genbank accession nos. AY530294, AY530296, AY530297 and AY530295, respectively. There is 100% identity between DSM 8442 and DSM 8443 at the 16S rRNA gene sequence level (Fig. 2). The high level of genetic similarity among these *P. l. pulvifaciens* strains suggested that they are clonally related. The sequence of DSM 3615 showed only 88% homology to DSM 8442/8443. However, a 100% identity was observed to *P. l. larvae* strain DSM 7030 partial 16S rRNA gene sequence. The 16S rDNA analysis showed once more

Table 2A
Carbohydrate metabolic profile of *P. larvae* using the api 50CH-system

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
carbohydrate	GLYcerol	ERYthrit	D ARAbinose	L ARAbinose	RIBose	D XYLose	L XYLose	ADOnit	β Methyl-D-Xyloside	GALactose	GLUcose	FRUctose	MaNnosE	SorBose	RHAmnose	DULcitol	INOsitol
P. l. larvae DSM 7030	+	-	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-
P. l. pulvifaciens DSM 3615	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-
P. l. pulvifaciens DSM 8442	+	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-	-
P. l. pulvifaciens DSM 8443	+	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-	-

	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
carbohydrate	MANnitol	SORbitol	α-Methyl-D-Mannoside	α-Methyl-D-Glucoside	N-Acetyl-Glucosamine	AMYgdalin	ARButin	ESCulin	SALicin	CELlobiose	MALtose	LACtose	MELibiose	SACcharose	TREhalose	INUlin	MeLeZitose
P. l. larvae DSM 7030	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-
P. l. pulvifaciens DSM 3615	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-
P. l. pulvifaciens DSM 8442	-	-	-	-	+	-	+	+	+	-	+	-	-	-	+	-	-
P. l. pulvifaciens DSM 8443	-	-	-	-	+	-	+	+	+	-	+	-	-	-	+	-	-

	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
carbohydrate	RAFfinose	AMiDon	GLYcogen	XyLiTol	GENtiobiose	D TURanose	D LYXose	D TAGatose	D FUCose	L FUCose	D ARAbitol	L ARAbitol	GlucoNaTe	2-Keto-Gluconate	5-Keto-Gluconate
P. l. larvae DSM 7030	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. l. pulvifaciens DSM 3615	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. l. pulvifaciens DSM 8442	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. l. pulvifaciens DSM 8443	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The carbohydrate metabolic pattern of all four reference strains of *P. larvae* (DSM 7030, DSM 3615, DSM 8442 and DSM 8443) were analyzed using the api 50CH-system. Positive reactions indicating the ability to metabolize the corresponding carbohydrate are indicated by a plus sign.

Table 2B
Biochemical fingerprinting of *P. larvae* using the api 20E-system

	1	2	3	4	5	6	7	8	9	10	11	12
biochemical reaction (substrate)	**beta-galactosidase (OPNG)**	**arginine dihydrolase**	**lysine decarboxylase**	**ornithin decarboxylase**	**citrate utilization**	**H2S production (Na thiosulfate)**	**urea hydrolysis**	**deaminase (tryptophan)**	**indole production (tryptophan)**	**acetoin production (Na pyruvate)**	**gelatinase (charcoal gelatin)**	**nitrate reduction (potassium nitrate)**
***P. l. larvae* DSM 7030**	-	-	-	-	-	-	-	-	-	-	(-)	-
***P. l. pulvifaciens* DSM 3615**	-	-	-	-	-	-	-	-	-	-	+	-
***P. l. pulvifaciens* DSM 8442**	-	-	-	-	-	-	-	-	-	-	+	-
***P. l. pulvifaciens* DSM 8443**	-	-	-	-	-	-	-	-	-	-	+	-

The ability of all four reference strains of *P. larvae* (DSM 7030, DSM 3615, DSM 8442 and DSM 8443) to perform specific biochemical reaction was analyzed using the api 20E-system. A plus sign indicates the ability to perform the corresponding reaction a minus sign in brackets indicates weak reactivity.

the high similarity of DSM 7030 and DSM 3615, suggesting they belong to the same subspecies.

4. Discussion

Difficulties in differentiating between *P. l. larvae* and *P. l. pulvifaciens* are a common theme of most of the studies dealing with classifying or diagnosing these two subspecies. Here we present evidence that this complicacy is at least partly due to a wrong classification of one reference strain for *P. l. pulvifaciens*, referred to in this study as DSM 3615 (for synonyms used in the literature see Table 3).

The most prominent distinctive feature between the two subspecies is the presence or absence of catalase activity. *P. l. larvae*, is described as obligatory catalase negative (Heyndrickx et al., 1996). In accordance, reference strain DSM 7030 reacted catalase negative (data not shown). In contrast to *P. l. larvae*, *P. l. pulvifaciens* can be catalase negative but also weak, delayed catalase positive (Heyndrickx et al., 1996), what was the case for reference strain DSM 3615 (data not shown). *P. l. pulvifaciens* strains DSM 8442 and 8443 were weak but not delayed catalase positive (data not shown) what might lie within an acceptable spectrum of the catalase test. All four strains were Plagemann-positive, i.e. giant whips were formed upon sporulation (data not shown). Therefore, the classification of the reference strains correlated with the features catalase activity and occurrence of giant whips.

Despite this classification, all molecular and biochemical methods applied in our study suggest that *P. larvae* reference strains DSM 3615 (originally classified as *P. l. pulvifaciens*) and DSM 7030 (classified as type strain for *P. l. larvae*) show a high degree of similarity. In particular, comparison of the 16S rDNA sequences of all four *P. larvae* strains reveal 100% sequence identity for strains DSM 3615 and DSM 7030 and, therefore, both belong to the same subspecies, *P. l. larvae*, *P. l. pulvifaciens* reference strains DSM 8442 and DSM 8443 also showed 100% sequence identity but with only 88% 16S rDNA sequence homology to DSM 7030/DSM 3615 they clearly differ from *P. l. larvae*.

Table 2C
Metabolic profile of different *P. larvae* reference strains as determined by using the BIOLOG-system

well	A4	A5	A9	A10	A11	B1	B3	B5	B11	C2	C3	C4	C6	C11	D1	D2	D4
carbon source	dextrin	glycogen	Tween 80	N-acetyl-D-glucosamine	N-acetyl-β-D-mannosamine	L-arabinose	arbutin	D-fructose	α-D-glucose	lactulose	maltose	maltotriose	D-mannose	3-methyl-D-glucose	β-methyl-D-glucoside	α-methyl-D-mannoside	D-psicose
BIOLOG standard for *P. l. larvae*	-	(+)	-	+	(+)	-	-	-	(+)	-	-	-	(+)	(+)	(+)	-	-
P. l. larvae DSM 7030	-	-	-	+	(+)	-	-	-	(+)	-	-	-	-	-	(+)	-	-
P. larvae DSM 3615	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
P. l. pulvifaciens DSM 8442	-	-	-	+	-	+	-	-	(+)	-	-	-	(+)	-	-	-	+
P. l. pulvifaciens DSM 8443	(+)	-	(+)	+	(+)	+	+	+	-	(+)	(+)	+	(+)	-	-	(+)	+

well	D7	D8	E1	E2	E3	E5	E6	E8	E9	E11	E12	F6	F7	F9	F12	G1	G3
carbon source	D-ribose	salicin	D-tagatose	D-trehalose	turanose	D-xylose	acetic acid	β-hydroxybutyric acid	γ-hydroxybutyric acid	α-ketoglutaric acid	α-ketovaleric acid	pyruvic acid methyl ester	succinic acid mono-methyl ester	pyruvic acid	N-acetyl-L-glutamic acid	L-alaninamid	L-alanine
BIOLOG standard for *P. l. larvae*	-	-	-	+	(+)	-	-	-	-	-	-	(+)	-	(+)	-	-	(+)
P. l. larvae DSM 7030	-	-	-	+	-	-	-	-	-	-	-	(+)	-	(+)	-	-	-
P. larvae DSM 3615	-	-	-	(+)	-	-	-	-	-	-	-	(+)	-	+	-	-	-
P. l. pulvifaciens DSM 8442	+	-	(+)	(+)	-	+	+	-	+	+	+	-	-	+	-	+	-
P. l. pulvifaciens DSM 8443	+	(+)	-	+	-	+	(+)	(+)	+	+	+	(+)	(+)	+	(+)	-	(+)

well	G6	G8	G9	G10	G11	G12	H1	H2	H3	H4	H5	H6	H7	H8	H9	H11	H12
carbon source	L-glutamic acid	L-pyroglutamic acid	L-serine	putrescine	2,3-butanediol	glycerol	adenosine	2'-deoxy-adenosine	inosine	thymidine	uridine	adenosine-5'-monophosphate	thymidine-5'-monophosphate	uridine-5'-monophosphate	D-fructose-6-phosphate	D-glucose-6-phosphate	D,L-α-glycerol-phosphate
BIOLOG standard for *P. l. larvae*	-	-	-	-	-	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	-	-	-
P. l. larvae DSM 7030	-	-	-	-	-	+	(+)	(+)	(+)	+	(+)	(+)	(+)	(+)	-	-	-
P. larvae DSM 3615	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-
P. l. pulvifaciens DSM 8442	-	-	(+)	(+)	-	+	-	-	-	-	-	-	-	-	+	(+)	(+)
P. l. pulvifaciens DSM 8443	(+)	(+)	(+)	-	(+)	+	-	-	-	-	-	-	-	-	+	(+)	(+)

The metabolic profile of all four reference strains of *P. larvae* (DSM 7030, DSM 3615, DSM 8442 and DSM 8443) was analyzed using the BIOLOG system and compared to the standard profile for *P. l. larvae* as defined by the BIOLOG-software. A plus sign or a plus sign in brackets indicates that 100% or less than 100%, respectively, of all strains investigated were able to metabolize the corresponding carbon source.

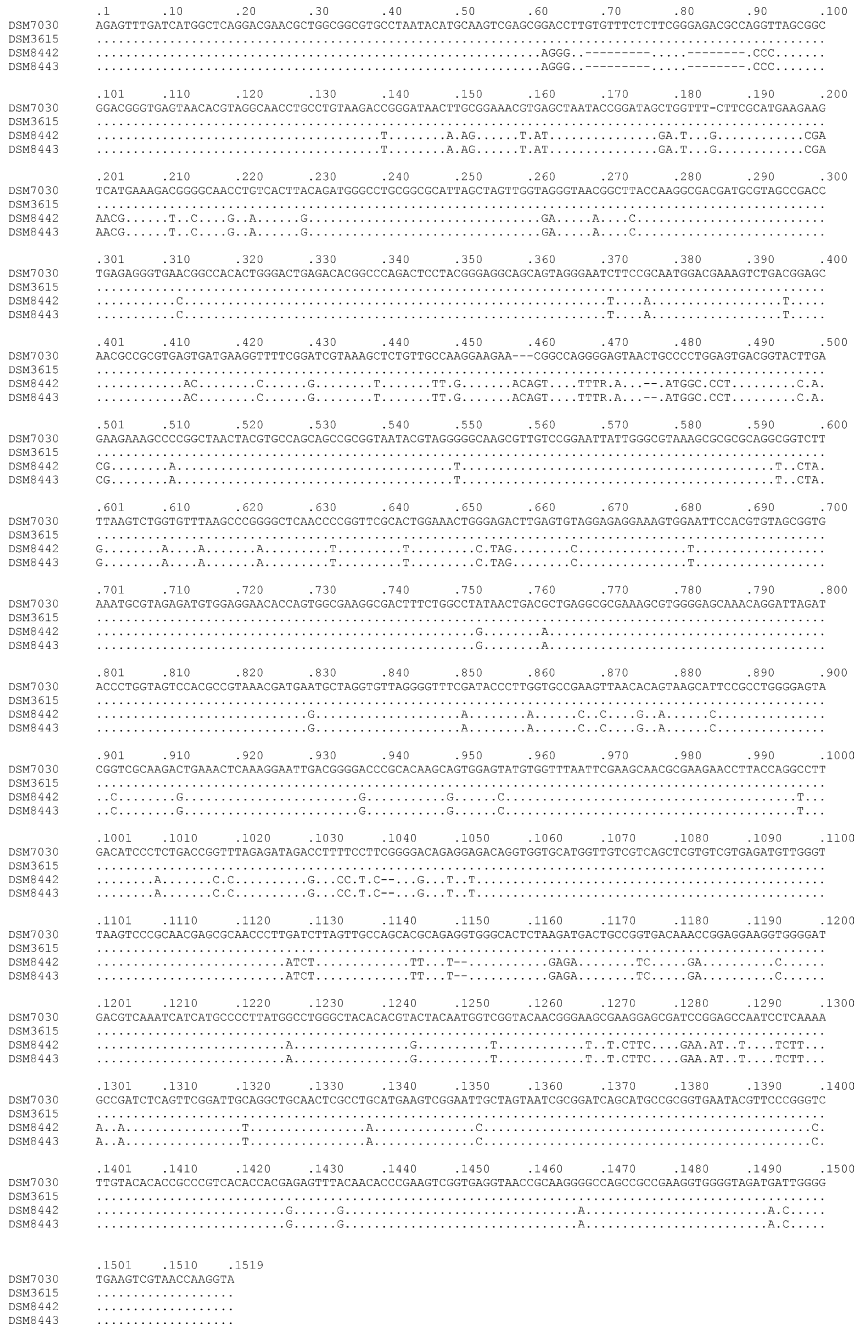

Fig. 2. Comparison of 16S rDNA sequences of *P. larvae*.

So far, attempts to establish a specific diagnostic PCR-based detection method for *P. l. larvae* failed because it appeared to be impossible to differentiate between the two subspecies *P. l. larvae* and *P. l. pulvifaciens* (Govan et al., 1999; Dobbelaere et al., 2001b; Lauro et al., 2003). In disaccord, our PCR results obtained with primers amplifying regions of the 16S rDNA and of a specific metalloprotease gene

Table 3
Synonymous names and corresponding references for *P. larvae* reference strains DSM 7030 and DSM 3615

Synonyms for DSM 7030	Referred to as *P. l. larvae* in
ATCC 9545	Ash et al. (1993)
	Heyndrickx et al. (1996)
	Lauro et al. (2003)
LMG 15969	Heyndrickx et al. (1996)
	Dobbelaere et al. (2001)
LMG 9820	Heyndrickx et al. (1996)
	Govan et al. (1999)
	Dobbelaere et al. (2001)
Synonyms for DSM 3615	**Referred to as *P. l. pulvifaciens* in**
ATCC 13537	Alippi et al. (2002)
ATCC 49843	Ash et al. (1993)
	Heyndrickx et al. (1996)
	Lauro et al. (2003)
LMG 15974	Dobbelaere et al. (2001b)
NRRL B-3670	Alippi et al. (2002)
NRRL B-3688	Alippi et al. (2002)
NRRL B-3685	Heyndrickx et al. (1996)
LMG 6911	
NCIMB 11201	

revealed that only *P. l. pulvifaciens* strain DSM 3615 was indistinguishable from *P. l. larvae* DSM 7030. Since our sequencing results indicate a 100% sequence identity for the 16S rRNA gene of DSM 3615 and *P. l. larvae* DSM 7030, it is consequential that these two strains cannot be distinguished by PCR-protocols based on amplifying 16S rRNA gene segments. More over, based on our sequence data we propose to reclassify *P. l. pulvifaciens* DSM 3615 as *P. l. larvae*.

Using the api 50CH-system, Heyndrickx et al. (1996) reported that *P. l. larvae* and *P. l. pulvifaciens* can be differentiated by their ability to metabolize mannitol and salicin: *P. l. larvae* metabolizes salicin (may be weak) but not mannitol, whereas *P. l. pulvifaciens* is able to use mannitol but not salicin. In agreement with other studies (Jelinski, 1985, Carpana et al., 1995; Dobbelaere et al., 2001a) our data do not confirm that the metabolism of mannitol or salicin can be used as a distinctive feature. Even though, using the api 50CH-system a characteristic metabolic pattern could be obtained for *P. l. larvae* (Carpana et al., 1995), studies analyzing the biochemical properties of *P. l. larvae* revealed a high degree of heterogeneity in the metabolic profile when different isolates are compared (Jelinski, 1985; Carpana et al., 1995; Dobbelaere et al., 2001a; Neuendorf et al., 2004). Further investigations are necessary to verify the potential of the widely used api-system to discriminate between the *P. larvae* subspecies. The only data available on the metabolic profile of *P. l. pulvifaciens* are based on analyzing strains LMG 6911 and LMG 15974 (Heyndrickx et al., 1996). However, LMG 6911 and LMG 15974 are synonyms for DSM 3615 (Table 3), which in turn shows many identical features in comparison to *P. l. larvae* DSM 7030 according to our extensive studies. Therefore, it is not surprising that the metabolic differentiation of the two subspecies as defined by Heyndrickx et al. (1996) did not endure after more detailed analysis.

In contrast to the api-system, the BIOLOG-software is able to identify *P. l. larvae*. The heterogeneous metabolic properties of *P. l. larvae* are reflected in the BIOLOG-standard profile for *P. l. larvae*. Interestingly, neither mannitol nor salicin, are given as potential carbon sources for *P. l. larvae*. When analyzing the four *P. larvae* reference strains, strains DSM 7030 and DSM 3615 were clearly identified as *P. l. larvae*, whereas *P. l. pulvifaciens* strains DSM 8442 and DSM 8443 obtained no identification. This result again indicates that *P. larvae* strain DSM 3615 indeed belongs to the subspecies *P. l. larvae* rather than to *P. l. pulvifaciens*.

In conclusion, based on our data both *P. larvae* strains DSM 7030 and DSM 3615 belong to the same subspecies *P. l. larvae* and, therefore, are indistinguishable by molecular methods. Evaluation of PCR-based methods using these two strains as reference strains for *P. l. larvae* and *P. l. pulvifaciens* (Dobbelaere et al., 2001b; Lauro et al., 2003) will consequentially result in the incorrect notion that PCR methods are unsuitable to differentiate between these two subspecies. Since 16S rDNA sequences of *P. l. pulvifaciens* strains DSM 8442 and DSM 8443 show only 88% homology to the sequences of *P. l. larvae* it is no problem to differentiate between these *P. l. pulvifaciens* strains and *P. l. larvae* by PCR-based methods. Biochemical fingerprinting using the BIOLOG system identified *P. larvae* strains DSM 7030 and DSM 3615 as *P. l. larvae* while strains DSM 8442 and DSM 8443 could not be identified by the software. Therefore, also the BIOLOG system, although indirectly, allows discrimination between *P. l. larvae* and *P. l. pulvifaciens*.

A diagnostic problem might arise from the fact that representatives of *P. larvae* strain DSM 3615 react weak, delayed catalase positive. Since *P. l. larvae*, is considered obligatory catalase negative such weak, delayed catalase positive isolates will be ruled out as *P. l. larvae* in routine diagnostics, producing false negative results. We, therefore, suggest that an emended description of *P. l. larvae* should include the possibility of a weak, delayed catalase-positive reaction and advise that analysis of *P. l. larvae*-suspect colonies should always include appropriate biochemical tests or PCR-based methods to clearly identify *P. l. larvae*.

Acknowledgements

The authors thank Daniela Reckling and Annette Beringhoff for excellent technical assistance. This work was supported by grants from the ministries for agriculture from Brandenburg and Sachsen-Anhalt, Germany.

References

Alippi, A.M., Lopez, A.C., Aguilar, O.M., 2002. Differentiation of *Paenibacillus larvae* subsp. *larvae*, the cause of American foulbrood of honeybees, by using PCR and restriction fragment analysis of genes encoding 16S rRNA. Appl. Environ. Microbiol. 68, 3655–3660.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Ash, C., Priest, F.G., Collins, M.D., 1993. Molecular identification of rRNA group 3 bacilli (Ash, Farrows, Wallbanks and Collins) using a PCR probe test. Antonie van Leeuwenhoek 64, 253–260.

Bailey, L., Ball, B.V., 1991. Honey Bee Pathology, 2nd edn. Academic Press, New York, London.

Buchholz-Cleven, B., Rattunde, B., Straub, K., 1997. Screening for genetic diversity of isolates of anaerobic Fe(II)-oxidizing bacteria using DGGE and whole-cell hybridization. Syst. Appl. Microbiol. 20, 301–309.

Carpana, E., Marocchi, L., Gelmini, L., 1995. Evaluation of the API 50CHB system for the identification and biochemical characterization of *Bacillus larvae*. Apidologie 26, 11–16.

Dobbelaere, W., de Graaf, D.C., Peeters, J.E., Jacobs, F.J., 2001a. Comparison of two commercial kits for biochemical characterization of *Paenibacillus larvae larvae* in the diagnosis of AFB. J. Apicult. Res. 40, 37–40.

Dobbelaere, W., de Graaf, D.C., Peeters, J.E., Jacobs, F.J., 2001b. Development of a fast and reliable diagnostic method for American foulbrood disease (*Paenibacillus larvae* subsp. *larvae*) using a 16S rRNA gene based PCR. Apidologie 32, 363–370.

Govan, V.A., Allsopp, M.H., Davison, S., 1999. A PCR detection method for rapid identification of *Paenibacillus larvae*. Appl. Environ. Microbiol. 65, 2243–2245.

Hansen, H., Brodsgaard, C.J., 1999. American foulbrood: a review of its biology, diagnosis and control. Bee World 80, 5–23.

Heyndrickx, H., Vandemeulebroecke, K., Hoste, B., Janssen, P., Kersters, K., de Vos, P., Logan, N.A., Ali, N., Berkeley, R.C.W., 1996. Reclassification of *Paenibacillus* formerly *Bacillus pulvifaciens*, 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. *pulvifaciens*. Int. J. Syst. Bacteriol. 46, 270–279.

Jelinski, M., 1985. Some biochemical properties of *Bacillus larvae* White. Apidologie 16, 69–76.

Katznelson, H., 1950. *Bacillus pulvifaciens* (n. sp.), an organism associated with powdery scale of honey-bee larvae. J. Bateriol. 59, 153–155.

Lauro, F.M., Favaretto, M., Rassu, M., Bertoloni, G., 2003. Rapid detection of *Paenibacillus larvae* from honey and hive samples with a novel nested PCR protocol. Int. J. Food Microbiol. 81, 195–201.

Neuendorf, S., Hedtke, K., Tangen, G., Genersch, E., 2004. Biochemical characterization of different genotypes of *Paenibacillus larvae* subsp. *larvae*, a honey bee bacterial pathogen. Microbiology 150, 2381–2390.

Plagemann, O., 1985. Eine einfache Kulturmethode zur bakteriologischen Identifizierung von *Bacillus larvae* mit Columbia-Blut-Schrägagar. Berl. Münch. Tierärztl. Wschr. 98, 61–62.

Ritter, W., 1996. Diagnostik und Bekämpfung von Bienenkrankheiten. Gustav Fischer Verlag, Jena.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.