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Biochemical characterization of different genotypes of *Paenibacillus larvae* subsp. *larvae*, a honey bee bacterial pathogen

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Paenibacillus larvae subsp. *larvae* (*P. l. larvae*) is the aetiological agent of American foulbrood (AFB), the most virulent bacterial disease of honey bee brood worldwide. In many countries AFB is a notifiable disease since it is highly contagious, in most cases incurable and able to kill affected colonies. Genotyping of field isolates of *P. l. larvae* revealed at least four genotypes (*AB*, *Ab*, *ab* and *αB*) present in Germany which are genotypically different from the reference strain DSM 7030. Therefore, based on these data, five different genotypes of *P. l. larvae* are now identified with genotype *AB* standing out with a characteristic brown-orange and circled two-coloured colony morphology. Analysing the metabolic profiles of three German genotypes (*AB*, *Ab* and *ab*) as well as of the reference strain using the Biolog system, a characteristic biochemical fingerprint could be obtained for each strain. Cluster analysis showed that while genotypes *Ab*, *ab* and the reference strain DSM 7030 are rather similar, genotype *AB* is clearly different from the others. Analysis of all isolates for plasmid DNA revealed two different plasmids present only in isolates belonging to genotype *AB*. Therefore, genotype *AB* is remarkable in all aspects analysed so far. Future analysis will show whether or not these differences will expand to differences in virulence.

INTRODUCTION

The spore-forming, Gram-positive bacterium *Paenibacillus larvae* subsp. *larvae* (*P. l. larvae*) is the causative agent of American foulbrood (AFB), the most serious and fatal bacterial disease of honey bee larvae. AFB is highly contagious and has spread around the world. In many countries, AFB is a notifiable disease and, hence, control measures are often regulated by disease control orders. Since destruction of infected colonies is considered the only workable control measure by most authorities, AFB is causing considerable economic loss to beekeepers worldwide.

The oval-shaped spores represent the infectious stage of *P. l. larvae*. AFB is transmitted by spore-containing honey being fed to newly hatched larvae. So far, the only known host for *P. l. larvae* is honey bee larvae. The spores germinate in the midgut lumen. The vegetative forms of *P. l. larvae* then penetrate the gut epithelium and proliferate within the larval tissue. Within 72 h the bee larvae are reduced to tissue detritus, which forms a glue-like colloid (rope stage). Later still the larval remains dry down to a scale adhering to the side of the cell. This scale is highly infectious since it contains billions of spores (Bailey & Ball, 1991; Gregorc & Bowen, 1998).

Abbreviations: AFB, American Foulbrood; rep-PCR, repetitive element PCR; *P. l. larvae*, *Paenibacillus larvae* subsp. *larvae*; *P. l. pulvifaciens*, *Paenibacillus larvae* subsp. *pulvifaciens*.

Recent genotyping of German field isolates of *P. l. larvae* revealed at least four different genotypes, named *AB*, *Ab*, *ab* and *αB* (Genersch & Otten, 2003). Here we present data on the further characterization of the genotypes *AB*, *Ab* and *ab*, and of the reference strain DSM 7030/ATCC 9545. Biochemical fingerprinting was performed using the Biolog system. This system involves the determination of the metabolism of 95 carbon sources in a microtitre plate format. Since the metabolism of bacteria is adapted to their natural environment or host, each bacterium prefers or uses particular carbon sources. Hence, determination of the metabolic profile of a micro-organism can be used for identifying and characterizing the organism.

Earlier reports show that some isolates of *P. l. larvae* harbour plasmid DNA (Benada *et al.*, 1988; Bodorova-Urgosikova *et al.*, 1992). Therefore, we analysed all isolates for the presence of extrachromosomal DNA to see whether or not the occurrence of plasmid DNA might be an additional feature suitable for typing purposes and epidemiology.

METHODS

Bacterial isolates. The *P. l. larvae* reference strain DSM 7030 was 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.

Paenibacillus larvae subsp. *pulvifaciens* (*P. l. pulvifaciens*) reference strains DSM 8442 and DSM 8443 were obtained from DSMZ. *P. l. pulvifaciens* DSM 8442 and DSM 8443 are synonyms for *P. l. pulvifaciens* reference strains NRRL NRS 1683 and NRRL NRS 1684, respectively.

Eighty-six German field isolates from *P. l. larvae* were isolated from honey samples originating from 86 AFB-positive hives diagnosed in the course of foulbrood monitoring programs between 2000 and 2003 (Table 1). Diagnosis was based on clinical symptoms as well as on isolation, cultivation and absolute identification of the causative agent, *P. l. larvae*. Honey samples had been stored at 4 °C until *P. l. larvae* was cultivated for scientific purposes from these samples on bacterial plates. Two representatives of the recently identified *P. l. larvae* genotype *AB* (Genersch & Otten, 2003) have been deposited at the DSMZ (accession numbers DSM 16115, DSM 16116).

Bacterial culture. *P. l. pulvifaciens* reference strains DSM 8442 and DSM 8443 as well as *P. l. larvae* reference strain DSM 7030 were cultured on Columbia sheep blood agar plates exactly as directed by DSMZ.

The culture of *P. l. larvae* from honey samples was performed essentially as previously described (Genersch & Otten, 2003). Briefly, for growth of spore-forming bacteria, honey samples were solubilized overnight at 37 °C. Subsequently, samples were diluted in double-distilled water to obtain a 50 % (w/v) honey solution. To select for spores, samples were incubated at 90 °C for 6 min. Samples were allowed to cool down at room temperature prior to plating them (200 µl per plate) onto Columbia sheep blood agar plates. 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, *P. l. larvae*-like colonies were identified by catalase and Plagemann tests as well as by PCR detection prior to subsequent detailed analysis.

For further analysis, all isolates were stored as bacterial suspensions in 25 % glycerol in BHI (brain heart infusion) broth at −70 °C.

Biochemical analysis of *P. l. larvae* reference strain DSM 7030 was performed by subculturing this strain and taking 24 independent subcultures.

Catalase and Plagemann tests. For absolute identification, colonies with a *P. l. larvae*-like morphology were further analysed by catalase and Plagemann tests. For the catalase test part of the colony in question was transferred to a microscopic slide using a 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 indicates a lack of catalase activity. For the Plagemann test (Plagemann, 1985), the liquid part of Columbia sheep blood agar slants was inoculated with part of the bacterial colony in question. The tube was air-tight sealed with Parafilm and incubated at 37 °C for 10 days. Subsequently, the liquid part was analysed for the presence of spores and giant whips by phase-contrast microscopy. *P. l. larvae* is characterized by the lack of catalase activity and giant whips occurring during sporulation (Ritter, 1996; Hansen & Brodsgaard, 1999).

P. l. larvae*-specific PCR.** For PCR identification of bacterial colonies grown on agar plates, part of the colony in question was resuspended in 50 µl double-distilled 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 ATCC 9545) and on the partial sequence of the gene for a 35 kDa metalloprotease from *P. l. larvae* (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′-AAACCGGTCAGAGGGATGTCAAG-3′; Pll-16S F6, 5′-GCACTGGAAACTGGGAGACTTG-3′; Pll-16S B11, 5′-CGGCTT-TTGAGGATTGGCTC-3′; Pll-MP F3, 5′-CGGGCAGCAAATCGTAT-TCAG-3′; Pll-MP B1, 5′-CCATAAAGTGTTGGGTCCTCTAAGG-3′.

PCR analyses were carried out in a final volume of 25 µl consisting of 1 × Qiagen reaction buffer, 250 µM dNTPs (dATP, dCTP, dGTP, dTTP), 10 µM primer and 0·3 U HotStarTaq polymerase (Qiagen). Concentrations of $MgCl_2$ were adjusted so that all three reactions could be performed at a final annealing temperature of 56 °C: 2·3 mM for primer pair Pll-16S E1/E2, 1·7 mM for primer pair Pll-16S F6/B11 and 1·5 mM for primer pair Pll-MP F3/B1. After the initial activation step (15 min, 95 °C), the reaction conditions for the touch-down PCR were as follows. All denaturation steps were performed at 94 °C for 30 s, all elongation steps were performed at 72 °C for 30 s, and all annealing steps were performed for 1 min. For annealing, temperatures of 66, 62 and 58 °C were used and 5 cycles were run at each temperature. At the final annealing temperature of 56 °C, 30 cycles were run followed by a final elongation step at 72 °C for 8 min. Five microlitres of the PCR samples was analysed on a 0·8 % agarose gel. The DNA bands were stained with ethidium bromide and visualized by UV light.

The expected lengths of specific amplicons generated with Pll-16S E1/E2, Pll-16S F6/B11 and Pll-MP F3/B1 were 965, 665 and 273 bp, respectively. For all *P. l. larvae* genotypes, specificity of amplicons was verified by sequencing (Medigenomix, Germany). When tested for specificity with *P. l. pulvifaciens* reference strains DSM 8442 and DSM 8443, none of the primers generated any PCR product (Fig. 1). Specific PCR products were not generated when control PCR analyses were performed with reference strains for *Paenibacillus alvei* (DSM 29), *Paenibacillus apiarius* (DSM 5581, DSM 5582, DSM 5612), *Paenibacillus polymyxa* (DSM 36), *Bacillus licheniformis* (DSM 13), *Bacillus mycoides* (DSM 299), *Bacillus thuringiensis* (DSM 6029) and other unidentified bacilli isolated from honey samples (data not shown).

Repetitive element PCR (rep-PCR) analysis. Preparation of bacterial DNA for fingerprinting and subsequent rep-PCR analysis of bacterial isolates was performed essentially as described previously (Genersch & Otten, 2003). In brief, *P. l. larvae* DNA suitable for rep-PCR DNA fingerprinting was isolated from colonies grown on culture plates using 6 % InstaGene matrix (Bio-Rad) following the instructions of the manufacturer. The DNA sequences of the primers used for DNA fingerprinting were as follows (Versalovic *et al.*, 1994): 5́-CTACGGCAAGGCGACGCTGACG-3 (BOX A1R), 5́-CCGCCGTTGCCGCCGTTGCCGCCG-3́ (MBO REP1). PCR analyses were carried out in a final volume of 25 µl consisting of 1 × reaction buffer (Qiagen) and a final concentration of 2·5 mM $MgCl_2$, 250 µM dNTPs (dATP, dCTP, dGTP, dTTP), 10 µM primer and 0·3 U HotStarTaq 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 were run followed by a final elongation step at 72 °C for 10 min. Five microlitres of the PCR samples was analysed on a 0·8 % agarose gel. The DNA bands were stained with ethidium bromide and visualized by UV light.

Biochemical fingerprinting (Biolog system). The Biolog system (obtained through Oxoid) is a carbon source test, where the ability of a bacterial isolate to metabolize 95 different carbon sources is used for identification purposes. Cultivation and preparation of *P. l. larvae* isolates for metabolic analysis using the Biolog system were 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*. Briefly, single pure colonies of

Table 1. List of isolates used in this study

All field isolates from *P. l. larvae* were isolated from honey originating from AFB-positive hives diagnosed in the course of foulbrood monitoring programs in Germany between 2000 and 2003. Honey samples had been collected close to the brood nest from brood combs showing clinical symptoms of AFB. DSM 7030 is a *P. l. larvae* reference strain (identical to *P. l. larvae* strain ATCC 9545) obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany).

Isolate	*P. l. larvae* genotype				Year of isolation	Isolate	*P. l. larvae* genotype				Year of isolation
	AB	***Ab***	***ab***	***aβ***			**AB**	***Ab***	***ab***	***aβ***	
00-775.1	+				2000	02-122		+			2002
00-775.2	+				2000	02-180		+			2002
00-869	+				2000	03-019		+			2003
00-897	+				2000	03-159		+			2003
00-1163.1	+				2000	00-087			+		2000
00-1163.2	+				2000	01-145			+		2001
01-000.1	+				2001	01-247			+		2001
01-000.2	+				2001	01-248			+		2001
01-000.3	+				2001	01-249			+		2001
01-649.1	+				2001	01-281			+		2001
01-649.2	+				2001	01-282			+		2001
01-1714	+				2001	01-283			+		2001
02-009	+				2002	01-337			+		2001
02-334	+				2002	01-391			+		2001
03-016.1	+				2003	01-402			+		2001
03-016.2	+				2003	01-454			+		2001
03-098	+				2003	01-455			+		2001
03-195	+				2003	01-456			+		2001
03-199	+				2003	01-1707.1			+		2001
03-201	+				2003	01-1707.2			+		2001
03-476	+				2003	01-1709.1			+		2001
03-478	+				2003	01-1709.2			+		2001
03-479	+				2003	02-127			+		2002
03-518	+				2003	02-128			+		2002
03-522	+				2003	02-129			+		2002
03-525	+				2003	02-130			+		2002
01-358		+			2001	02-141			+		2002
01-440		+			2001	02-149			+		2002
02-060		+			2002	02-179			+		2002
02-065		+			2002	02-250			+		2002
02-066		+			2002	02-360			+		2002
02-067		+			2002	03-015			+		2003
02-070		+			2002	03-017			+		2003
02-075		+			2002	03-121			+		2003
02-076		+			2002	03-122			+		2003
02-081		+			2002	03-125a			+		2003
02-083		+			2002	03-125b			+		2003
02-108		+			2002	03-125c			+		2003
02-111.1		+			2002	03-125d			+		2003
02-111.2		+			2002	03-126			+		2003
02-113		+			2002	03-128			+		2003
02-117		+			2002	03-384			+		2003
02-120		+			2002	DSM 7030				+	Reference strain
02-121		+			2002						

P. l. 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. Using sterile wooden sticks a special streaking technique was performed resulting in a 'plus' sign on the centre of the plate. The goal of this technique is to restrain cell growth to two thin lines so that the cells along the edges

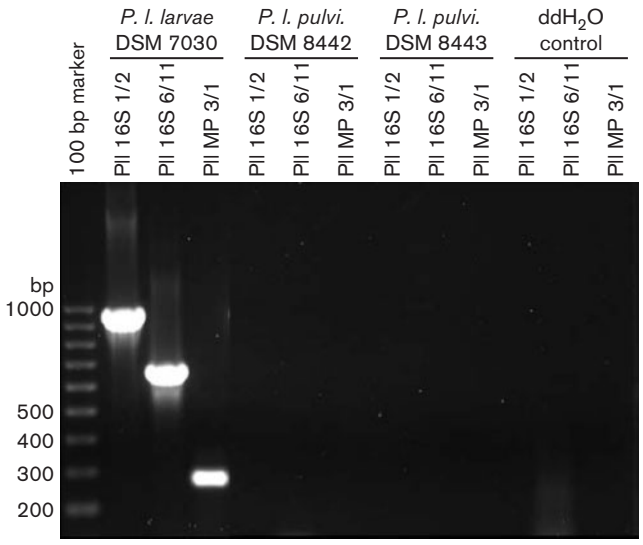

Fig. 1. PCR identification of *P. l. larvae*. PCR analysis of *P. l. larvae* reference strain DSM 7030 and *P. l. pulvifaciens* reference strains DSM 8442 and DSM 8443 using primer pairs PII-16S E1/E2 (PII 16S 1/2) and PII-16S F6/B11 (PII 16S 6/11) for amplification of 16S rDNA fragments and primer pair PII-MP F3/B1 (PII MP 3/1) for amplification of a metalloproteinase gene segment. PCR products were analysed on a 0·8 % agarose gel in the presence of appropriate molecular size markers.

have a good supply of food. This keeps the cells in an active state and decreases sporulation. For the analysis of one isolate, eight pure colonies of this isolate were subcultured on eight BUG-M-T plates. Growth of bacteria at 37 °C was continued for 48 h since *P. l. larvae* is a slow-growing bacterium. Subsequently, the inoculum was prepared by taking only those colonies starting at the ends of the 'plus' sign to half way down the junction of the two lines constituting the 'plus' sign. Bacteria from the centre or close to the centre must not be taken. Colonies were picked up with a wooden stick and rubbed around the walls of an empty, sterile dry glass tube. The bacterial film was suspended in 5 ml inoculation solution (GN/GP IF; Oxoid) to obtain a homogeneous mixture. After adding the remaining fluid (10 ml), turbidity was adjusted to match the turbidity standard (Oxoid) at 28±2 % turbidity (OD=0·55). Subsequently, a GP2 MicroPlate (Oxoid) 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 (end point 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 after choosing plate type (GP2), strain type (GP-ROD SB) and incubation time (16–24 h). Interpretation of the results and identification of the bacterial strain in question was performed automatically by the software. The Biolog database does include a standard profile for *P. l. larvae* (Table 2) and, therefore, is able to identify *P. l. larvae*. The software does not include *P. l. pulvifaciens*.

Cluster analysis was performed by Biolog MicroLog3 software after manually entering the data. The dendrogram was generated using a modified UPGMA analysis (Biolog) where the algorithm uses DIST values to generate the branching structure of the dendrogram.

Preparation of plasmid DNA. Frozen bacterial suspensions were thawed, plated on Columbia sheep blood agar plates and allowed to grow for 3 days at 37 °C. Colonies were scraped off and resuspended in 300 µl BHI broth. Up to six plates were pooled to yield a sufficient amount of bacteria for plasmid preparation. Subsequently, bacteria were pelleted by centrifugation at 5000 ***g*** for 10 min. The bacterial pellet was used for plasmid preparation performed with the QIAprep Spin Miniprep kit (Qiagen) by exactly following the manufacturer's protocol. A 12 µl sample of each eluent was analysed on a 0·8 % agarose gel. The DNA bands were stained with ethidium bromide and visualized by UV light.

Restriction analysis of plasmid DNA. Depending on band intensity, 2–8 µl of each eluent containing plasmid DNA was used for restriction analysis. Restriction reactions were carried out in a final volume of 10 µl using 10 U of the corresponding restriction enzyme together with the appropriate reaction buffer. Reactions were incubated at 37 °C for 30 min. Subsequently, 2 µl 6× DNA loading buffer was added and the reactions were analysed on a 0·8 % agarose gel. The DNA bands were stained with ethidium bromide and visualized by UV light.

RESULTS

rep-PCR analysis of *P. l. larvae*

By analysing German *P. l. larvae* isolates using rep-PCR fingerprinting performed with primers BOX A1R and MBO REP1, three BOX A1R and two MBO REP1 patterns (*A*, *a*, *α* and *B*, *b*, respectively) were characterized recently (Genersch & Otten, 2003). Differential combinations of these patterns were found, resulting in the identification of the four genetic subgroups *AB*, *Ab*, *ab* and *αB* (Genersch & Otten, 2003). rep-PCR analysis of *P. l. larvae* reference strain DSM 7030 (identical to ATCC 9545) now revealed a third MBO REP1 pattern (*β*), with the characteristic band around 1000 bp missing, and its affiliation to BOX A1R-group *a* (Fig. 2). Hence, *P. l. larvae* DSM 7030 belongs to a new genotype *aβ*, hitherto not found as a field isolate in Germany.

Colony morphology of *P. l. larvae*

Colonies of *P. l. larvae* subgroups *Ab*, *ab* and *aβ* have a whitish to greyish, somewhat transparent and slightly glistening appearance (Fig. 3a). In contrast, isolates belonging to subgroup *AB* show a considerable deviation from this normal colony morphology. Small colonies of *AB* are unicoloured red-brown or white without being transparent, whereas bigger colonies mostly show concentric brownish and whitish circles with the outermost circle always being orange-brown (Fig. 3b). Both the unicoloured and the circled colony phenotypes were unambiguously identified as *P. l. larvae* by Plagemann and catalase tests as well as by *P. l. larvae*-specific PCR detection. Both phenotypes have identical rep-PCR patterns characteristic for subgroup *AB* (data not shown). The phenotypic differences are not stable in the sense that serial cultivation of single white, single red-brown and single brownish-circled colonies would result in only white, red-brown and brownish-circled colonies, respectively. These variants rather split up again into the

Table 2. Metabolic profiles of different strains of *P. l. larvae* as determined by using the Biolog system

Results are expressed as a percentage of positive reactions obtained for each carbon source. Only those carbon sources used by *P. l. larvae* are given.

Well:	A5	A10	A11	B5	B11	C6	C11	D1	D4	E2	E3	F6	F9	G3	G12	H1	H2	H3	H4	H5	H6	H7	H8
Carbon source:	Glycogen	*N*-Acetyl-D-glucosamine	*N*-Acetyl-*β*-D-mannosamine	D-Fructose	*α*-D-Glucose	D-Mannose	3-Methyl-D-glucose	*β*-Methyl-D-glucoside	D-Psicose	D-Trehalose	Turanose	Pyruvic acid methyl ester	Pyruvic acid	L-Alanine	Glycerol	Adenosine	2′-Deoxy-adenosine	Inosine	Thymidine	Uridine	Adenosine-5′-monophosphate	Thymidine-5′-monophosphate	Uridine-5′-monophosphate
Biolog standard	5	100	15	0	50	20	20	20	0	100	15	70	90	10	100	20	5	10	20	30	10	15	20
Strain DSM 7030 ($n=24$)*	0	100	13	0	54	0	0	38	0	100	0	75	96	0	100	58	21	17	100	58	50	83	79
Genotype *AB* ($n=26$)†	0	100	19	100	58	38	0	0	96	92	0	96	100	0	0	69	73	0	96	96	0	0	0
Genotype *Ab* ($n=22$)†	0	100	0	0	86	0	0	27	0	100	0	77	100	0	100	86	91	59	100	100	100	100	100
Genotype *ab* ($n=38$)†	0	100	0	0	50	0	0	24	0	100	0	97	100	0	87	97	97	84	100	87	95	100	97

**P. l. larvae* reference strain DSM 7030 was obtained from the DSMZ culture collection. Twenty-four independent subcultures of this strain were used for Biolog characterization to obtain a value for 'percentage of positive reactions'.

†*P. l. larvae* genotypes *AB*, *Ab* and *ab* were isolated from different outbreaks of AFB in Germany between 2000 and 2003. Different and independent field isolates (*n* given in brackets) were used for Biolog characterization to obtain the percentage of positive reactions.

same three variants (data not shown). Therefore, not only the deviation, but also the variability of colony pigmentation has to be considered as a stable and characteristic morphological feature of colonies of subgroup *AB*.

Biochemical characterization of *P. l. larvae*

The Biolog system takes into account that the metabolic profile of a given bacterium is not a static feature but rather differs between different isolates or independent cultures of one isolate. Hence, the standard profiles are given as percentages of positive reactions for each carbon source. Using the Biolog system (Oxoid) we analysed the metabolic pattern of *P. l. larvae* subgroups *AB*, *Ab*, *ab* and *aβ* (Table 2). Genotypes *AB*, *Ab* and *ab* were represented by 26, 22 and 38 different and independent field isolates, respectively. To also obtain ratios of positive reactions for genotype *aβ*, *P. l. larvae* reference strain DSM 7030 was tested by taking 24 independent subcultures of this strain. All 110 samples were unambiguously identified as *P. l. larvae* by the Biolog identification software. The standard metabolic pattern established for *P. l. larvae* by Biolog and used for identification is also given in Table 2 (Biolog standard). All cultures of strain DSM 7030 (genotype *aβ*) were able to metabolize *N*-acetyl-D-glucosamine, D-trehalose, glycerol and thymidine, whereas D-fructose, D-mannose, 3-methyl-D-glucose, D-psicose, turanose and L-alanine were not accepted as carbon source. The other carbon sources given in the table were differentially used by DSM 7030. All isolates of genotype *AB* were able to metabolize *N*-acetyl-D-glucosamine, D-fructose and pyruvic acid, but none was able to degrade 3-methyl-D-glucose, *β*-methyl-D-glucoside, turanose, L-alanine, glycerol, inosine, adenosine-5′-monophosphate, thymidine-5′-monophosphate and uridine-5′-monophosphate. The other carbon sources given in the table were used by 19–96 % of all *AB* isolates. All isolates of genotypes *Ab* and *ab* were able to digest *N*-acetyl-D-glucosamine, D-trehalose, pyruvic acid, thymidine and thymidine-5′-monophosphate. In addition, 100 % of genotype *Ab* metabolized glycerol, uridine, adenosine-5′-monophosphate and uridine-5′-monophosphate. *N*-Acetyl-*β*-D-mannosamine, D-fructose, D-mannose, 3-methyl-D-glucose, D-psicose, turanose and L-alanine were never used by any isolate of genotypes *Ab* and *ab*. All other carbon sources given in the table were differentially metabolized by isolates belonging to these subgroups. The

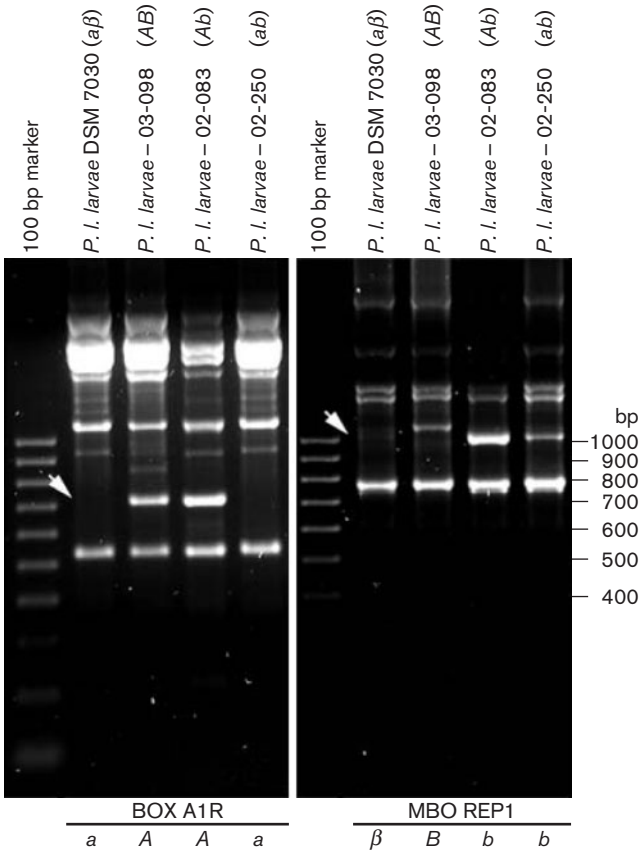

Fig. 2. *P. l. larvae* reference strain DSM 7030 belongs to the newly identified genotype *aβ*. Using BOX A1R and MBO REP1 primers, *P. l. larvae* reference strain DSM 7030 was analysed by rep-PCR. For comparison, rep-PCR patterns from recently identified *P. l. larvae* genotypes *AB*, *Ab* and *ab* are also shown. Differences in banding pattern between the BOX groups *A* and *a* and between the MBO groups *β*, *B* and *b* are highlighted by arrows. By combining the different BOX and MBO patterns for a single isolate the four genetic subgroups *aβ*, *AB*, *Ab* and *ab* are revealed.

relationships between the four different analysed strains in comparison to the Biolog standard profile and other metabolically related bacteria are given in the dendrogram in Fig. 4. Genotypes *Ab*, *aβ* and *ab* are rather similar to each other, but nevertheless distinct from the Biolog standard metabolic profile. The metabolic distance between genotype *AB* and the other genotypes becomes abundantly clear.

Screening for and characterization of plasmid DNA harboured by *P. l. larvae*

Inspired by earlier reports on some *P. l. larvae* isolates harbouring plasmids (Benada *et al.*, 1988; Bodorova-Urgosikova *et al.*, 1992; Drobnikova *et al.*, 1994), we screened all our isolates for extrachromosomal DNA. We found two different plasmid molecules of 9·4 and 11·0 kb, denoted pPll9.4 and pPll11.0, respectively (Fig. 5). The

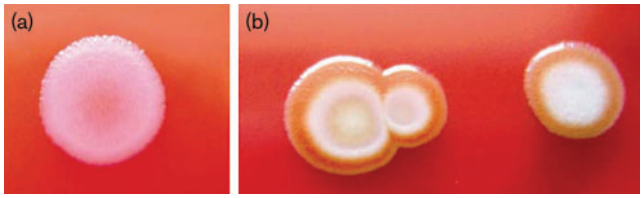

Fig. 3. Characteristic colony morphology of *P. l. larvae*. (a) The normal morphology of colonies of *P. l. larvae* is whitish to greyish, somewhat transparent and slightly glistening. (b) In contrast, colonies of genotype *AB* show orange-brown circles and clearly differ from normal colony morphology. Colonies shown are 6 days old.

presence of plasmids was restricted to isolates belonging to genotype *AB*; no plasmid DNA could be found in the reference strain DSM 7030 or in *Ab* and *ab* strains (Fig. 5). Normally, one isolate harboured either pPll9.4 or pPll11.0, but in one case both plasmids were found together (Fig. 5, lane 7). The characteristic restriction cleavage pattern of the predominant pPll9.4 obtained with restriction enzymes *Hin*dIII, *Eco*RI and *Xba*I is shown in Fig. 6(a). As estimated from the electrophoretic mobilities, *Hin*dIII generates six fragments of 500, 600, 1150, 2300, 2400 and 2450 bp, the latter two migrating as a double band. In contrast, *Eco*RI generates only two fragments of 850 and 8550 bp. Plasmid pPll9.4 is linearized by *Xba*I resulting in a band migrating at 9400 bp, correlating with the size of the plasmid as calculated from the sum of the fragments generated with *Hin*dIII and *Eco*RI. Plasmid pPll11.0

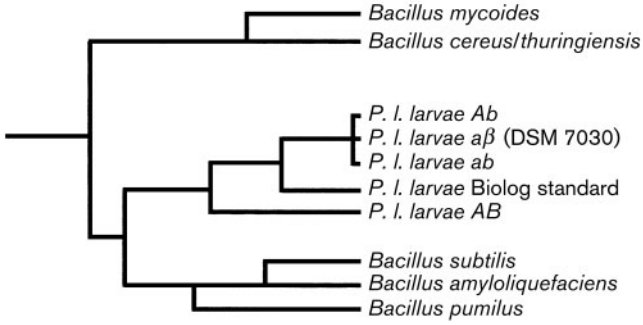

Fig. 4. Dendrogram showing the metabolic relationship between *P. l. larvae* genotypes *Ab*, *aβ*, *ab* and *AB* in relation to the Biolog standard and other metabolically related bacilli. Multiple samples of the different genotypes were analysed for their metabolic profile using the Biolog system. Based on the results obtained, a characteristic metabolic pattern for each genotype could be defined. These characteristic profiles were used to construct a dendrogram via a modified UPGMA analysis (Biolog). Genotypes *Ab*, *aβ* and *ab* are distinct, but nevertheless quite similar. In contrast, genotype *AB* is clearly different from all other genotypes as well as from the Biolog standard.

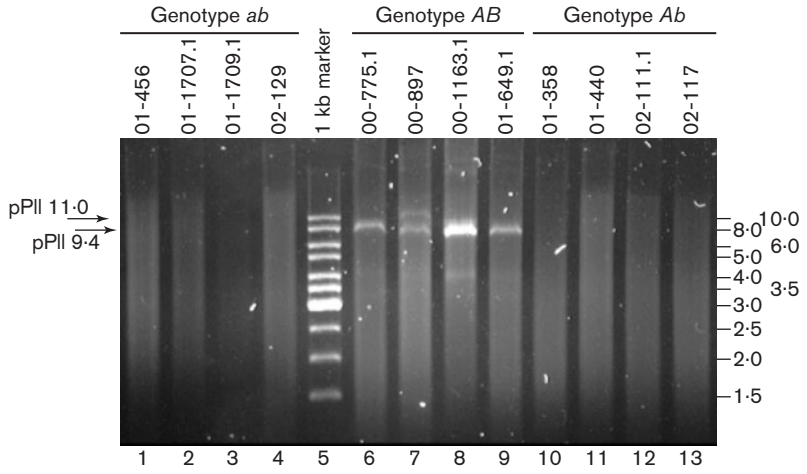

Fig. 5. Plasmids isolated from *P. l. larvae*. All isolates analysed in detail in this study were also screened for the presence of extrachromosomal DNA. Isolates belonging to genotype *AB* harboured two different plasmids of 9·4 and 11·0 kb, named pPll9.4 and pPll11.0, respectively. No plasmid DNA could be isolated from the other genotypes. Representative results are shown.

obviously results from a 1600 bp insertion into the 2400 bp *Hin*dIII fragment which is part of the 8550 bp *Eco*RI fragment as can be deduced from comparing the restriction cleavage patterns of pPll9.4 and pPll11.0 (Fig. 6b). This insertion contains an additional *Xba*I site since cleavage of pPll11.0 with *Xba*I does not linearize the plasmid but generates two fragments of 6000 and 5000 bp.

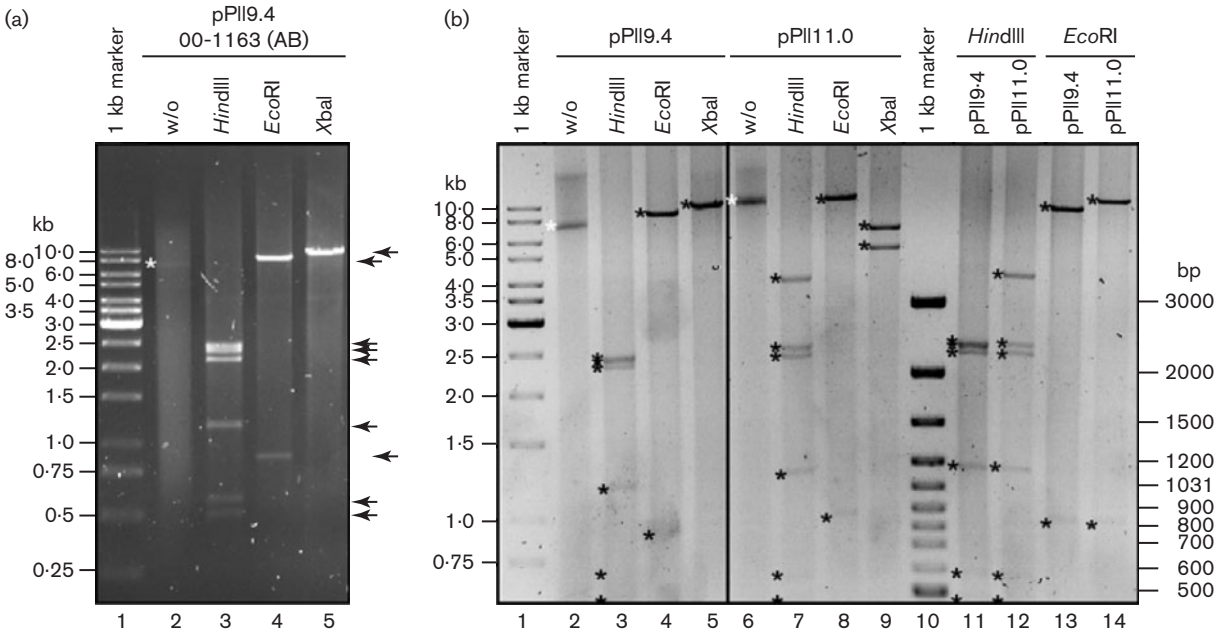

Fig. 6. Restriction analysis of plasmids pPll9.4 and pPll11.0. (a) Restriction analysis of plasmid pPll9.4 was performed with restriction enzymes *Hin*dIII, *Eco*RI and *Xba*I. Cleavage with *Hin*dIII yielded six fragments of 500, 600, 1150, 2300, 2400 and 2450 bp (lane 3). Obviously, pPll9.4 has only two *Eco*RI sites giving rise to two fragments of 850 and 8550 bp (lane 4). *Xba*I linearized pPll9.4; accordingly, the *Xba*I fragment of pPll9.4 migrates around 9400 bp (lane 5). (b) Analysing both plasmids pPll9.4 and pPll11.0 by restriction enzyme cleavage revealed that for all three enzymes the restriction patterns of pPll9.4 (lanes 2–5) and pPll11.0 (lanes 6–9) show differences resulting from a 1600 bp insertion into the 2400 bp *Hin*dIII fragment (lanes 11, 12) and into the 8550 bp *Eco*RI fragment (lanes 13, 14) respectively. In addition, the insertion contains a second *Xba*I site, leading to two *Xba*I fragments of 6000 and 5000 bp (lane 9) as opposed to the single *Xba*I fragment of pPll9.4 (lane 5). The *Hin*dIII double band of pPll9.4, migrating around 2450 bp, as well as fragments smaller than 900 bp, are hardly visible in (b) but clearly visible in (a). w/o, No enzyme.

DISCUSSION

AFB is the most serious and fatal bacterial disease of honey bee larvae. It is caused by the spore-forming bacterium *P. l. larvae*. Absolute identification of *P. l. larvae* requires the bacterium be grown in the laboratory (Office International des Epizooties, 2000). Diagnosis of AFB then relies on visually identifying *P. l. larvae* colonies grown on agar plates. Only suspect colonies are further analysed to confirm the initial identification. Normal colony morphology of *P. l. larvae* is described as whitish to greyish, somewhat transparent and slightly glistening (Bailey & Ball, 1991). In this study, we demonstrated that the above description does not hold true for all isolates of *P. l. larvae*. Genotype *AB*, identified among German field isolates (Genersch & Otten, 2003), is characterized by a striking circled whitish and brownish or unicoloured red-brown or white morphology. Although a pigmented variant of *P. l. larvae* has been described (Drobnikova *et al.*, 1994), in more recent studies production of an orange pigment has only been attributed to *P. l. pulvifaciens* (Heyndrickx *et al.*, 1996). Here, we again provide evidence for the existence of a pigmented phenotype of *P. l. larvae*. While Drobnikova *et al.* (1994) failed to find any feature discriminating their pigmented variant from the non-pigmented strains, we can show that the ability to produce pigmented colonies is characteristic for *P. l. larvae* genotype *AB*. The importance of this observation lies within the fact that not knowing this particular phenotype might lead to false-negative diagnostic results. False-negative diagnostic results are a threat to beekeeping since AFB is highly contagious and is able to kill affected colonies. Control measures for AFB depend on correct and early diagnosis of this disease. Therefore, knowledge of all possible phenotypic variations of *P. l. larvae* colonies is vital for exact diagnosis.

Earlier reports on biochemical characterization of *P. l. larvae* using traditional macro (Jelinski, 1985; Alippi & Aguilar, 1998) or commercial micro methods (Carpana *et al.*, 1995; Dobbelaere *et al.*, 2001) showed the usefulness of such tests for the classification of *P. l. larvae*, although the results obtained were somewhat contradictory. It was suggested that these differences are due to the different systems used (Dobbelaere *et al.*, 2001). Here we present evidence that the discrepancies between different studies on the biochemical properties of *P. l. larvae* are rather due to genotype-specific differences. We analysed the metabolic pattern of four different genotypes of *P. l. larvae* (*AB*, *Ab*, *ab* and *aβ*) by using the Biolog system. The system involves the determination of the metabolism of 95 different carbon sources. Interpretation of positive reactions is performed via an ELISA reader. Allotting the metabolic fingerprints to the different genotypes revealed characteristic patterns for each genotype (Table 2). To our knowledge this is the first time that genotype-specific metabolic profiles could be defined for *P. l. larvae*, indicating that differences in genotype correlate with differences in biochemical phenotype. When compared to other genotypes, *P. l. larvae* genotype *AB* exhibits the most striking metabolic pattern, since it is the only strain able to metabolize the carbohydrates D-fructose (100 %) and D-psicose (96 %), and the only strain unable to use glycerol as carbon source. The other strains, of genotypes *Ab*, *ab* and *aβ*, also show characteristic metabolic patterns, but are nevertheless more similar to each other. Analysing the metabolic pattern of two reference strains for *P. l. pulvifaciens*, DSM 8442 and DSM 8443, resulted in no identification using the Biolog software, since their biochemical fingerprint differed in more than 20 carbon sources from the profiles of *P. l. larvae* (J. Kilwinski, M. Peters, A. Ashiralieva & E. Genersch, unpublished results). Therefore, the Biolog system allows not only the identification of *P. l. larvae*, but also the definite discrimination between genotype *AB* and the other genotypes. Since the Biolog system is used in microbiological diagnosis, this result will be of diagnostic relevance if differences in virulence can be assigned to differences in genotype.

Our results show that the characteristic metabolic patterns always contain some variables for each genotype. Hence, groupings based on biochemical properties where these properties are understood as static traits will lead to results which hardly correlate with genotyping.

Comparing our results with the Biolog standard we can conclude that at least genotype *AB* was not included when establishing the standard metabolic fingerprint for *P. l. larvae*. D-Fructose and D-psicose are both given 0 %, meaning that it was never accepted as a carbon source by any isolate included in the survey. In contrast, D-fructose and D-psicose are metabolized by 100 and 96 %, respectively, by isolates belonging to genotype *AB*. Furthermore, some strains included in the Biolog standard are not represented in our study, since we never found any isolate able to metabolize 3-methyl-D-glucose, turanose or L-alanine. The exact metabolic relationships between the different genotypes of *P. l. larvae* in relation to the Biolog standard are given in the dendrogram (Fig. 4). The distance in biochemical phenotype between genotype *AB* and the other genotypes and, in contrast, the relative closeness of genotypes *Ab*, *ab* and *aβ* become obvious.

Jelinski (1985) distinguished seven biochemical types (I–VII) according to seven possible combinations of three biochemical properties: reduction of nitrate to nitrite, hydrolysis of mannitol and acid production from salicin. The same study revealed the ability to metabolize glycerol as a consistent feature of *P. l. larvae*. Based on our analysis using the Biolog system, this holds true for genotypes *Ab*, *ab* and the reference strain DSM 7030. In contrast, no isolate belonging to genotype *AB* was able to use glycerol as carbon source. It has been reported that comparison between the biochemical type (I–VII; Jelinski, 1985) and the genotype of isolates rendered no obvious link between both features (Alippi & Aguilar, 1998). This result is in disagreement with our results showing a clear link between biochemical and rep-PCR fingerprints. In the study performed by Alippi & Aguilar (1998) genotyping is based on rep-PCR performed with primers BOX A1R and REP (REP1R.I and REP2-I), a

primer combination having less discriminatory power than BOX A1R combined with MBO REP1 (Genersch & Otten, 2003), as used in our study. Therefore, it is likely, that the genotypes defined by Alippi & Aguilar (1998) would split up if MBO REP1 primers were used instead of REP primers, possibly allowing a better correlation between biochemical type and genotype. Furthermore, the discriminatory power of only three metabolic properties is quite poor as compared to the Biolog system where the metabolism of a total of 95 carbon sources is analysed. Above all, if the three metabolic properties chosen by Alippi & Aguilar (1998) are variable features within the genotypes it will be impossible to find any obvious linkage between both features.

Based on the API 50CHB system, Carpana *et al.* (1995) determined the ability of *P. l. larvae* to metabolize 49 carbohydrates and their derivatives and presented a detailed comparison of the results with those reported in the literature. In particular for galactose, fructose and mannitol the results were contradictory. We found that among the isolates investigated in our study only genotype *AB* was able to metabolize D-fructose and mannose, for example. Therefore, we propose that the discrepancies between different studies are in part due to different genotypes analysed in these studies that in turn might be due to the differential geographic origin of the isolates.

Our data show that genotype *AB* is outstanding in respect to colony morphology and metabolic fingerprint. When screening all isolates used in this study for extrachromosomal DNA, only representatives of genotype *AB* were found to harbour plasmids. So far, no plasmid DNA has been detected in isolates from genotypes *Ab*, *ab* or *aβ*. Therefore, another characteristic feature of genotype *AB* is the presence of plasmids. The occurrence of plasmid DNA in *P. l. larvae* has been reported (Benada *et al.*, 1988; Bodorova-Urgosikova *et al.*, 1992; Drobnikova *et al.*, 1994). The plasmid denoted pBL423/728 is about 9·4 kb in size and this seems to be in agreement with our data at first. But whereas plasmid pBL423/728 does not contain an *Xba*I restriction site and digestion with *Eco*RI results in two fragments of 3·6 and 5·8 kb (Bodorova-Urgosikova *et al.*, 1992), plasmid pPll9.4 characterized in our study is linearized by *Xba*I and gives rise to two *Eco*RI fragments of 850 and 8550 bp. Hence, the two plasmids, although similar in size, are not identical. Since pPll11.0 differs from pPll9.4 only by a 1600 bp insertion, this plasmid also is not related to plasmid pBL423/728.

It has long been recognized that the proteins comprising the parasporal Cry toxins of *Bacillus thuringiensis*, an insecticidal, Gram-positive, spore-forming bacterium, are generally encoded by large plasmids (Gonzalez *et al.*, 1981; for review see Schnepf *et al.*, 1998). Nothing is known so far about toxins expressed by *P. l. larvae*. It will be interesting to screen the newly found plasmids of *P. l. larvae* for genes possibly involved in pathogenicity.

In some countries, *P. l. larvae* has been treated in bee colonies by the antibiotic oxytetracyclin for several decades. Recently, widespread resistance to oxytetracyclin has been reported (e.g. Miyagi *et al.*, 2000). A recent study analysing the origin of oxytetracyclin resistance in *P. l. larvae* did not include any search for plasmids, but rather focused on the correlation between 16S rDNA haplotypes and resistance (Evans, 2003). No convincing correlation was found, thus leading to the speculation that resistance might be epigenetic in nature, specifically through the presence of plasmids and mobile genetics entities that produce proteins involved in resistance (Adams *et al.*, 1998). Although no specific resistance to sulphonamides, antibiotics, mercury chloride or cadmium nitrate connected with the presence of plasmid pBL423/728 in *P. l. larvae* was found (Benada *et al.*, 1988), this is still an open question for pPll9.4 and pPll11.0. Since both these plasmids are not related to pBL423/728 it will be interesting to further characterize the newly identified plasmids and look for any resistance-connected genes carried by those plasmids.

Overall, our study has identified and characterized the exceptional *P. l. larvae* genotype *AB* for the first time. Considering what is known so far about this genotype, it may be the first choice for more detailed analyses with respect to virulence, pathogenicity and antibiotic resistance.

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