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METHODS

A PCR-Based Method for Distinguishing between Two Common Beehive Bacteria, *Paenibacillus larvae* and *Brevibacillus laterosporus*

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ABSTRACT *Paenibacillus larvae* and *Brevibacillus laterosporus* are two bacteria that are members of the *Paenibacillaceae* family. Both are commonly found in beehives and have historically been difficult to distinguish from each other due to related genetic and phenotypic characteristics and a shared ecological niche. Here, we discuss the likely mischaracterization of three 16S rRNA sequences previously published as *P. larvae* and provide the phylogenetic evidence that supported the GenBank reassignment of the sequences as *B. laterosporus*. We explore the issues that arise by using only 16S rRNA or other single-gene analyses to distinguish between these bacteria. We also present three sets of molecular markers, two sets that distinguish *P. larvae* from *B. laterosporus* and other closely related species within the *Paenibacillus* genus and a third set that distinguishes *B. laterosporus* from *P. larvae* and other closely related species within the *Brevibacillus* genus. These molecular markers provide a tool for proper identification of these oft-mistaken species.

IMPORTANCE 16S rRNA gene sequencing in bacteria has long been held as the gold standard for typing bacteria and, for the most part, is an excellent method of taxonomically identifying different bacterial species. However, the high level of 16S rRNA sequence similarity of some published strains of *P. larvae* and *B. laterosporus*, as well as possible horizontal gene transfer events within their shared ecological niche, complicates the use of 16S rRNA sequence as an effective molecular marker for differentiating these two species. Additionally, shared characteristics of these bacteria limit the effectiveness of using traditional phenotypic identification assays, such as the catalase test. The results from this study provide PCR methods to quickly differentiate between these two genera and will be useful when studying *Brevibacillus*, *Paenibacillus*, and other disease-relevant bacteria commonly found in beehives.

KEYWORDS beehive, *Brevibacillus*, PCR, *Paenibacillus*

Brevibacillus laterosporus and *Paenibacillus larvae* are two bacteria that share several genotypic and phenotypic similarities and can therefore be difficult to distinguish from one another (1–5, 57). Both species belong to the family *Paenibacillaceae* (6), were formerly classified in the genus *Bacillus* (7), and can be isolated from both healthy and diseased beehives (8, 9). *P. larvae* infection can cause American foulbrood (AFB) (10) in honeybee populations, while *B. laterosporus* is distributed broadly among many insect populations and can act as a mutualistic probiotic or an antagonistic pathogen (11). *B. laterosporus* can act as a secondary invader in beehives following infection by *Melissococcus plutonius*, which causes European foulbrood (12). In some cases, *B. laterosporus* appears to be antagonistic toward *P. larvae* (11, 13, 14). Additionally, certain strains of *Brevibacillus* can produce pharmacologically relevant molecules like spergualin, which has demonstrated antitumor properties in mouse leukemia and antibiotic effects

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against certain *Bacillus* and *Staphylococcus* species (15, 16). Researchers working to detect *P. larvae* in sick beehives often inadvertently isolate *B. laterosporus* as well as other related bacteria (1–4). Therefore, additional methods for isolation, detection, and characterization are requisite for proper discrimination between these closely related genera at both the genotypic and phenotypic levels.

The close ecological and evolutionary ties of *Paenibacillus* and *Brevibacillus* have led to many reported difficulties distinguishing these strains (1–5, 57). The misidentification between the two species in at least two previous studies warrants a closer inspection of the methods used to identify and characterize these bacteria. Earlier publications reported the isolation (17) and characterization (18) of five *P. larvae* phages (Jimmer1, Jimmer2, Davies, Emery, and Abouo) using two field strains (previously named PL2 and PL6) isolated from beehives using protocols designed for specific isolation and selection of *P. larvae* spores (3, 17, 19). These field isolates initially appeared to be *P. larvae* based on previously established phenotypic assays (17, 20), including the absence of catalase activity and >97% sequence identity to the 16S rRNA genes of three isolates listed in NCBI databases as *P. larvae* or *P. larvae* subsp. *pulvifaciens* (accession numbers AY530296, AY530297, and FR877752). However, after further investigation, the previously identified *P. larvae* field isolates PL2 and PL6 were reclassified as *B. laterosporus* (21). Such corrections highlight the complexity of distinguishing between these bacteria. The close ecological and evolutionary ties of *P. larvae* and *B. laterosporus* necessitate the careful consideration of factors such as the complicated nature of *P. larvae* catalase testing (22), new protocols for *P. larvae* spore isolation (20), the recent addition of other *Paenibacillus* and *Brevibacillus* sequences to GenBank, and/or the sequence analysis of additional phages isolated using field isolates (21, 23). The results of this study address the need for more-definitive methods to distinguish between *P. larvae* and *B. laterosporus* than have been previously available.

In this report, shared characteristics complicating the correct identification of *Brevibacillus* and *Paenibacillus* are presented and discussed. We show that several 16S rRNA genes sequenced from bacteria that were isolated from plants and beehives and deposited in NCBI are most likely incorrectly named and need to be reidentified as 16S rRNA genes belonging to *B. laterosporus*. In addition, we describe multilocus PCR-based methods that efficiently distinguish between *Brevibacillus* species and *P. larvae*, providing more concrete identification methods for these particular strains. The tools provided here will aid in the discrimination and study of *Paenibacillus*, *Brevibacillus*, and other beehive-associated bacteria, a wide field of international study due to the prevailing diseases afflicting honeybees.

RESULTS

Comparison of *Brevibacillus* and *Paenibacillus* 16S rRNA gene sequences reveals misclassification of several *B. laterosporus* strains. Researchers working to detect *P. larvae* in beehives often isolate *B. laterosporus* and other closely related bacteria due to insufficient discriminatory techniques for these similar species (1–5). Our field isolates Br1 to Br10 and Br14 were initially classified as *P. larvae* based on their isolation on selective media, the absence of catalase activity, PCR-amplified 16S rRNA sequence similarity to *P. larvae*, and other previously described tests (17, 20). BLASTN (24–26) results indicated that the partial 16S rRNA gene sequences of the 11 strains, previously reported as PL1 to PL10 and PL14 but here reclassified as Br1 to Br10 and Br14 (18), shared an average of 97% identity (range, 93 to 99%) with the 16S rRNA gene from two *P. larvae* subsp. *pulvifaciens* strains DSM 8442 and DSM 8443 (AY530296.1 and AY530297.1, respectively), but also 97% identity (range, 93 to 99%) with the 16S rRNA gene from a wide variety of *Brevibacillus* strains, including *B. laterosporus* strain B9 (CP011074.1). These data highlight the shortcomings of relying on selective isolation techniques and single-gene analysis when isolating some bacteria. These strains' sequences were equally similar to 16S rRNA sequences of both *P. larvae* and *B. laterosporus* but due to their phenotypic characteristics were classified as *P. larvae*. Both the Ribosomal Database Project (RDP) and Greengenes were used to further analyze

TABLE 1 Bacterial genomes used in phylogenetic analyses[a]

Species	BGSC no.	Original name	16S rRNA gene accession no.	Reference or source
Paenibacillus alvei	33A1	*Paenibacillus alvei* III_3DT-1A	KF597222	49
Paenibacillus sp.	35A1	*Paenibacillus* sp. JDR-2	KF597225	50
Paenibacillus polymyxa	25A2	NRRL B-4317^T	KF597214	51
Paenibacillus larvae	Not in BGSC	ATCC 9545	NR_118956.1	52
Paenibacillus larvae	Not in BGSC	DSM 7030	NR_042947.1	53
Paenibacillus larvae	Not in BGSC	BD12OL1-B15	FR877752.1	NA
Paenibacillus larvae subsp. *pulvifaciens* 73	Not in BGSC	DSM 8442	AY530296	53
Paenibacillus larvae subsp. *pulvifaciens* 73	Not in BGSC	DSM 8443	AY530297	53
Staphylococcus aureus	Not in BGSC	ATCC 12600	NR_118997.1	54
Brevibacillus brevis	26A1	ATCC 8246	D78457.1	55
Brevibacillus laterosporus	Not in BGSC	LMG 15441	CP007806.1; 43591, 437436*	56
B. laterosporus	Not in BGSC	B9	CP011074; 1091175, 1092715*	58
B. laterosporus	40A1	ATCC 9141	KF597228	42, 59
B. laterosporus	Not in BGSC	Br1	NA	This study
B. laterosporus	Not in BGSC	Br2	NA	This study
B. laterosporus	Not in BGSC	Br3	NA	This study
B. laterosporus	Not in BGSC	Br4	NA	This study
B. laterosporus	Not in BGSC	Br5	NA	This study
B. laterosporus	Not in BGSC	Br6	NA	This study
B. laterosporus	Not in BGSC	Br7	NA	This study
B. laterosporus	Not in BGSC	Br8	NA	This study
B. laterosporus	Not in BGSC	Br9	NA	This study
B. laterosporus	Not in BGSC	Br10	NA	This study
B. laterosporus	Not in BGSC	Br12	NA	This study
B. laterosporus	Not in BGSC	Br13	NA	This study
B. laterosporus	Not in BGSC	Br14	NA	This study
Brevibacillus sp.	Not in BGSC	*Brevibacillus* sp.	NA	This study

[a]Strains with a BGSC number were obtained from the Bacillus Genetic Stock Center (www.bgsc.org). *, genome accession number and coordinates (base pairs) for rRNA genes without individual accession numbers. NA, not available or not applicable.

16S rRNA sequences of these field isolates in a broader phylogenetic context and study the ambiguity surrounding these isolates. Both of these databases placed every sequenced 16S rRNA gene from the field isolates (Br1 to Br10 and Br14) unambiguously in the *Brevibacillus* genus with close similarities (>97%) to many *B. laterosporus* strains. We also analyzed the 16S rRNA sequences of *P. larvae* strains DSM 8442 [AY530296.1], DSM 8443 [AY530297.1], and BD12OL1-B15 [FR877752.1] using RDP analysis. All three of these sequences yielded the same result as our field isolates in that they were identified as belonging to the genus *Brevibacillus* and had sequence matches to only *Brevibacillus* strains and to *P. larvae* strains DSM 8442 and DSM 8443.

To confirm these findings, phylogenetic analysis was performed on the 16S rRNA gene sequences from the 11 *Brevibacillus* isolates (Br1 to Br10 and Br14), three confirmed *P. larvae* strains, and one *Staphylococcus aureus* strain, which was used as an outgroup. These bacteria, along with others used in this study, are referenced in Table 1. Figure 1 is a neighbor-joining phylogenetic tree of these strains generated in MEGA7 (27) and aligned using MUSCLE (28). This tree indicates that the 16S rRNA gene sequences of field isolates, previously reported as *P. larvae* (now Br1 to Br10 and Br14), as well as *P. larvae* subsp. *pulvifaciens* DSM 8442 and DSM 8443 and *P. larvae* BD12OL1-B15, are most closely related to 16S rRNA gene sequences from confirmed *B. laterosporus* isolates. The findings from this phylogenetic analysis accurately represent the taxonomic relationships identified in the RDP and Greengenes database analyses. The recent increase of *P. larvae* and *B. laterosporus* rRNA sequence data has facilitated these observations regarding misclassification of *B. laterosporus* strains by at least three research groups studying *P. larvae* (including Br1 to Br10 and Br14 from our studies and *P. larvae* subsp. *pulvifaciens* DSM 8442 and DSM 8443 and *P. larvae* BD12OL1-B15 from others). We reported our concerns regarding strains DSM 8442, DSM 8443, and BD12OL1-B15 to GenBank, which resulted in the *P. larvae* subsp. *pulvifaciens* isolates

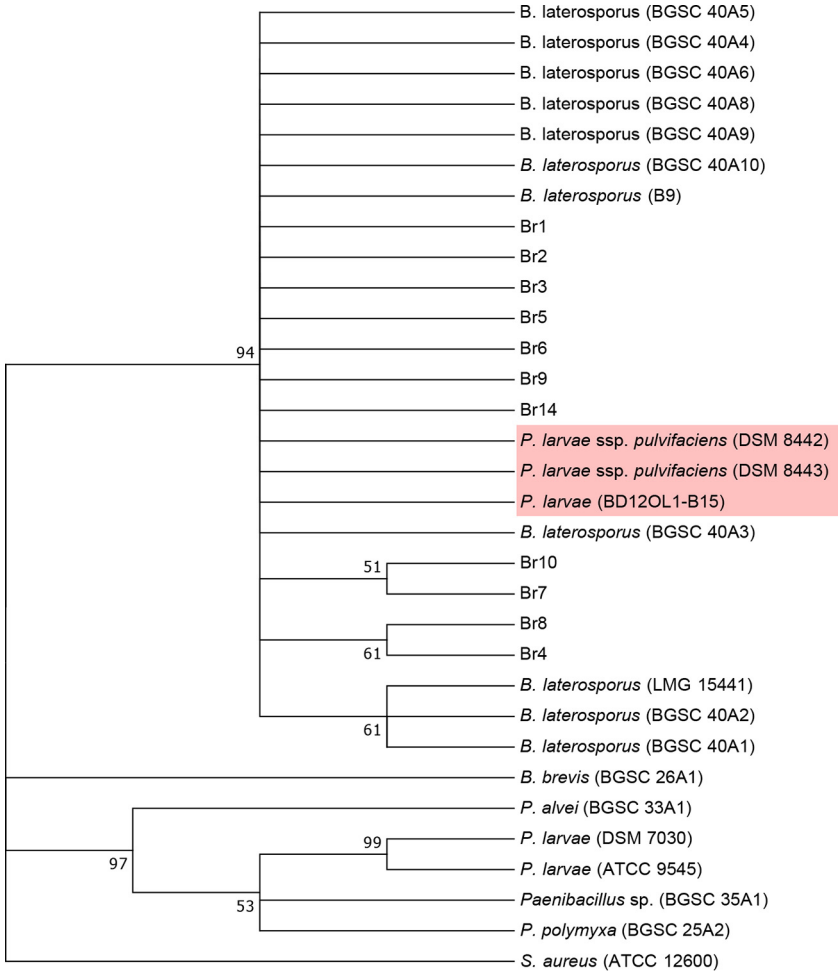

FIG 1 Phylogenetic tree distinguishing *Paenibacillaceae* strains by 16S rRNA genes. This tree indicates the relationships between *B. laterosporus* field isolates (Br), *B. laterosporus*, *B. brevis*, *P. alvei*, *P. polymyxa*, *Paenibacillus* sp., *P. larvae*, and *S. aureus* (outgroup). Reported *P. larvae* species that grouped with *Brevibacillus* bacteria are highlighted. The evolutionary distances were calculated using the maximum composite likelihood method (27) and are in the units of the number of substitutions per site. The evolutionary history was inferred using the neighbor-joining method (47) in MEGA7 (27) with a bootstrap value of 2,000 (48). The optimal tree with the sum of branch length of 0.48885562 is shown. Any branches with a bootstrapping score of less than 50% were collapsed.

(DSM 8442 and DSM 8443) being labeled as "unverified." These results also led to the reclassification of our field isolates as *B. laterosporus* (Br1 to Br14) (17). However, due to the genotypic, phenotypic, and ecological similarities of these two genera, as well as the reported frequencies of horizontal gene transfer within bacteria that share an ecological niche, reliance on a single gene to assign taxonomy can be perilous.

While the issues surrounding the phylogenetic classification of these Br strains have been solved by a closer look at the 16S rRNA sequences of several strains, single-gene analysis was at first insufficient to distinguish between these bacterial species or even their genera because of previous misclassifications of *B. laterosporus* strains as *P. larvae*. This created misleading genetic similarities among unrelated taxa at the 16S rRNA gene. In general, these single-locus similarities often cannot fully reflect whole-genome relationships due to the horizontal transfer of genes, especially between bacteria that share the same ecological niche (for a sampling of recent discussions on the issues surrounding the use of 16S rRNA sequencing and the necessity for analysis of multiple loci, see references 29–38).

Additional genetic markers are useful and at times necessary to remove ambiguity surrounding species classification. Thus, we set out to identify unambiguous targets

TABLE 2 Summary of *P. larvae* and *B. laterosporus* identification by PCR[a]

	P. larvae					*B. laterosporus*		
Strain	***rpoB***	***ftsA***	***thiG***	***Vagabond 1***	***Vagabond 2***	***rpoB***	***ftsA***	***thiG***
B. laterosporus								
40A1	+	−	−	−	−	^	+	+
Br1	+	−	−	−	~	^	+	+
Br2	^	−	−	−	−	+	+	+
Br3	^	−	−	−	−	^	+	+
Br4	+	−	−	−	−	^	+	+
Br5	+	−	−	−	−	^	+	+
Br6	^	−	−	−	−	+	+	+
Br7	^	−	−	−	−	^	+	+
Br9	^	−	−	−	−	+	+	+
Br12	+	−	−	NA	NA	+	+	−
Br13	+	−	−	NA	NA	+	+	+/−
Br14	^	−	−	−	−	+	+	+
B. brevis ATCC 8246	−	−	−	−	−	−	+	+/−
Brevibacillus sp.	−	−	−	NA	NA	−	−	−
P. larvae								
ATCC 9545	+	+	^	^	^	−	−	−
PL309	NA	NA	NA	^	^	NA	NA	NA
PL325	NA	NA	NA	^	^	NA	NA	NA
PL335	NA	NA	NA	^	^	NA	NA	NA
PL337	NA	NA	NA	^	^	NA	NA	NA
PL338	+	+	^	^	^	−	−	−
PL344	NA	NA	NA	^	+	NA	NA	NA
PL345	NA	NA	NA	+	+	NA	NA	NA
PL357	+	+	^	^	+	−	−	−
P. alvei	^	−	−	−	−	−	−	−
P. polymyxa	^	+	−	−	−	−	−	−
Paenibacillus sp.	^	−	^/−	−	−	−	~	−

[a]+, presence of the expected amplicon; ^, an occasional or consistent amplicon of an unexpected size generated in addition to the expected size for the primer pair due to off-target effects; −, lack of any amplicon as visualized by agarose gel electrophoresis; ~, an amplicon would be consistently or occasionally produced, but not of the expected size; +/−, an amplicon that would appear sporadically but that would disappear as the annealing temperature was increased a few degrees above the given annealing temperature (to 60°C); ^/−, occasionally no product, and when there is a product, there is an occasional or consistent amplicon of an unexpected size generated in addition to the expected size; NA, that particular strain was not used in testing the specified primer set. Each result listed was obtained from two or more independent reactions.

that could reliably distinguish between these bacteria at the species level. *B. laterosporus* field isolates Br1 to Br10 and Br14 were analyzed by PCR at additional loci (*rpoB*, *ftsA*, *thiG*, and other random ["*Vagabond*"] loci) to try to distinguish between *Brevibacillus laterosporus* and *Paenibacillus larvae* strains at the species level. The *rpoB* locus in *Brevibacillus laterosporus* and two *Vagabond* loci in *Paenibacillus larvae* are reliable in rapidly distinguishing these complicated species and do not require sequencing. A summary of the results can be found in Table 2.

PCR of *rpoB* is not sufficient for distinguishing *Paenibacillus* bacteria at the species level. Previously reported primers designed to target the *rpoB* gene in *P. larvae* (39) produced an ~400-bp amplicon in *P. larvae* ATCC 9545 as predicted. The expected amplicon size was also produced in the other *P. larvae* field isolates tested. *P. alvei*, *P. polymyxa*, and *Paenibacillus* sp. produced the ~400-bp amplicon but also produced an amplicon of unexpected size. In addition, these primers produced an ~400-bp band in all *B. laterosporus* field isolates as well as *B. laterosporus* 40A1. Six of the 11 Br field isolates also produced an unexpected ~200-bp band, likely the result of off-target binding. The single, expected ~400-bp amplicon from *P. larvae* ATCC 9545, as well as the single, unexpected ~200-bp amplicons from *B. laterosporus* field isolates Br2, Br6, and Br14, were sequenced. As expected, BLAST (24–26) results indicated that the sequenced ~400-bp amplicon from *P. larvae* ATCC 9545 was indeed the *rpoB* gene

TABLE 3 Primers used to differentiate *P. larvae* and *B. laterosporus* strains

Target gene	Primer sequence[a]	Direction	Primer concn (μM)	Annealing temp (°C)	Purpose	Expected size (bp)	Reference or source
16S rRNA	5′-AGAGTTTGATCMTGGCTCAG-3′	Forward	49.74	52	16S rRNA gene	~1,500	43
	5′-CCGTCAATTCMTTTRAGTTT-3′	Reverse	38.52		Universal primer		
P. larvae rpoB	5′-ATAACGCGAGACATTCCTAA-3′	Forward	67.8	52	Amplifies *P. larvae rpoB*	~400	39
	5′-GAACGGCATATCTTCTTCAG-3′	Reverse	82.8				
B. laterosporus rpoB	5′-GCAGGTAAACTGGTCCAGAGCG-3′	Forward	49.76	69.1	Amplifies *B. laterosporus rpoB*	~900	This study
	5′-CACCTGTTGATTTATCAATCAGCG-3′	Reverse	45.8				
P. larvae ftsA	5′-AAATCGGTGAGGAAGACATT-3′	Forward	52.8	52	Amplifies *P. larvae ftsA*	~500	39
	5′-TGCCAATACGGTTTACTTTA-3′	Reverse	74.6				
B. laterosporus ftsA	5′-CAGTCAAAATGATCTTATTGTCAGCATCG-3′	Forward	46.54	55	Amplifies *B. laterosporus ftsA*	~1,200	This study
	5′-CATCTTTTCAATGAAATTCCCTTGAG-3′	Reverse	48.68				
P. larvae thiG	5′-GCCAAGGCTTCGGGGCGGC-3′	Forward	47.42	65	Amplifies *P. larvae thiG*	~800	This study
	5′-CTCATTTATTTACTTCCAGACTGATGC-3′	Reverse	45.72				
B. laterosporus thiG	5′-GCATTCCTTCTATCGGACTACTAGC-3′	Forward	44	55+[b]	Amplifies *B. laterosporus thiG*	~775	This study
	5′-CGGATACGACTTTACGAATTGGAGAG-3′	Reverse	49.2				
P. larvae Vagabond 1	5′-CTGGATGTGGCCCAAACCTTTC-3′	Forward	36.3	65	Amplifies *P. larvae* genomic region	~1,500	This study
	5′-CCTGCACGGCAAAGGAAGGC-3′	Reverse	34.76				
P. larvae Vagabond 2	5′-AAAAAAAATAGTGAGGTTGGCACAG-3′	Forward	30.8	60	Amplifies *P. larvae* genomic region	~750	This study
	5′-CTGTGTAGAGAATGAATACGGAAT-3′	Reverse	44.06				

[a]M and R, degenerate bases in primer sequences.
[b]+, an occasional increase in the annealing temperatures (60°C) was needed in order to eliminate sporadic, spurious bands.

found in *P. larvae* (E value = 0; 99% identity). BLAST analysis of the sequenced unexpected ~200-bp amplicon from Br2, Br6, and Br14 indicated that this genetic segment is part of a hypothetical protein in *B. laterosporus* LMG 15441 (>95% identity; E value, <1e−28), supporting off-target amplification. These data indicated that the *P. larvae rpoB* primers are not sufficient to distinguish *P. larvae* from *B. laterosporus* under these PCR conditions because both *P. larvae* and *B. laterosporus* strains produced the expected 400-bp amplicon, along with confounding off-target amplicons.

PCR of *ftsA* or *thiG* cannot reliably distinguish *Paenibacillus* or *Brevibacillus* at the species level. Additional loci were tested for their ability to distinguish between *Brevibacillus* and *Paenibacillus*. The *Paenibacillus ftsA* primers, designed for a previous study (39), produced the expected ~500-bp amplicon in *P. larvae* ATCC 9545 and *P. larvae* field isolates PL338 and PL357, as well as in *P. polymyxa*. These primers also failed to produce an amplicon in all Br field isolates and all other *Brevibacillus* species (Table 2). While these markers were effective at distinguishing *Paenibacillus* at the genus level, they were incapable of classifying these strains at a species level as had been anticipated. For further differentiation among *Paenibacillus* strains, we refer investigators to a recent study by Douglas Dingman on genotyping *Paenibacillus* through the use of enterobacterial repetitive intergenic consensus (ERIC)-PCR and restriction fragment length polymorphism (RFLP) (5).

In search of new discriminatory targets, *thiG* was identified as a target with enough sequence uniqueness between these hosts to potentially be useful. Using the *Paenibacillus*-specific *thiG* primers, amplicons were produced that matched the expected size of ~800 bp as well as a lighter, occasional band of ~650 bp indicative of off-target binding in *P. larvae* ATCC 9545, in two verified *P. larvae* field isolates used in this study, PL338 and PL357, and occasionally in *Paenibacillus* sp. Amplicons of the expected size were not produced in any of the *Brevibacillus* species or strains or in any of the additional *Paenibacillus* species used in this study (Table 2; see also Fig. S2A in the supplemental material). Primers designed to amplify *thiG* therefore discriminate at the *Paenibacillus* genus level (Table 3). These results imply that both sets of these genus-specific primers (*ftsA* and *thiG*) would be useful in verifying whether a strain belongs to a particular genus, *Paenibacillus* or *Brevibacillus*, but they are unable to classify strains at the species level.

In order to complement the *Paenibacillus ftsA* primers, primers were designed to target *ftsA* in *Brevibacillus* (see Table 3). PCRs using these primers produced amplicons in all *B. laterosporus* field isolates, the BGSC (Bacillus Genetic Stock Center; www.bgsc.org) *B. laterosporus* strain (positive control), and *B. brevis* that were all ~1,200 bp in size, as expected. These primers failed to produce amplicons in any of the *Paenibacillus*

species or strains used in this study, except for what appeared to be off-site amplification in *Paenibacillus* sp., where faint bands of incorrect size (600-bp and 1,000-bp amplicons) were occasionally observed. Fig. S1 in the supplemental material shows a sample of the total *Brevibacillus*-specific *ftsA* PCR data; however, a complete listing of results can be found in Table 2. Thus, these primers were valuable in identifying *Brevibacillus* at the genus level but did not provide a species level marker for *B. laterosporus*.

Primers designed to amplify *thiG* were also able to identify most of the isolates belonging to the *Brevibacillus* genus (Tables 2 and 3). Using the *Brevibacillus*-specific *thiG* primers, the expected ~775-bp amplicons were produced in the BGSC *B. laterosporus* strain, all but one of the Br field isolates from this study (Br12 did not amplify, while Br13 would amplify most of the time), and at times *B. brevis*. As these primers were designed for Br field isolates, it is logical that the amplicon produced by *B. brevis* was, at times, elusive and could be eliminated by applying a higher annealing temperature (60°C or higher). These primers failed to produce an amplicon of any size in *Brevibacillus* sp. or in any of the *Paenibacillus* species (Fig. S2B; Table 2). Based on these results, both sets of these primers (for *ftsA* and *thiG*) might be useful in verifying whether a strain belongs to the *Brevibacillus* genus but do not appear to be able to distinguish between certain species within their genera and at times seem to have trouble identifying all *Brevibacillus* strains (in the case of the *B. laterosporus thiG* primers).

While the *B. laterosporus* and *P. larvae* primers discussed above would be useful in verifying whether a given strain belongs to the *Brevibacillus* or *Paenibacillus* genus, they do not provide the ability to distinguish between species and produce confounding off-target amplicons. As such, these experiments further demonstrate the difficulty and danger in using single-gene analysis to identify strains at the species level.

***Vagabond* PCR primers for *P. larvae* and *rpoB* primers for *B. laterosporus* can effectively discriminate at both the genus and species levels.** In order to identify additional loci specific for *P. larvae* at the species level, we first identified genomic regions of various sizes that were conserved across *P. larvae* and then compared these highly conserved regions against other *Paenibacillus* and *Brevibacillus* strains to ensure that they were unique to *P. larvae*. These methods are outlined in Materials and Methods. As a result, both of these molecular markers ("*Vagabond*" primers) for *P. larvae* were successful in amplifying only the expected amplicon in *P. larvae* and no other species tested. A band of approximately 1,500 bp using *P. larvae Vagabond 1* primers (Fig. 2A) or approximately 750 bp using *P. larvae Vagabond 2* primers (Fig. 2B) was amplified in *P. larvae* ATCC 9545, PL309, PL325, PL335, PL337, PL338, PL344, PL345, and PL357 with an occasional additional band in some of these strains. No amplification was observed in other *Paenibacillus* strains such as *Paenibacillus* sp., *P. polymyxa*, and *P. alvei* (Table 2). Additionally, no spurious bands were observed in any *Brevibacillus* genus members except occasionally in Br1 using *P. larvae Vagabond 2* primers. These loci were successful at distinguishing *P. larvae* from any closely related *Paenibacillus* species such as *P. polymyxa* and *P alvei* and from all *Brevibacillus* strains.

An additional primer was designed to be able to more reliably discriminate between *B. laterosporus* at the species level by targeting an ~900-bp segment of the *rpoB* gene in *B. laterosporus* (Table 3). All Br field isolates and *B. laterosporus* 40A1 (positive control) produced the predicted ~900-bp amplicon (Fig. 3 shows a sample of the total PCR data for the *Brevibacillus*-specific *rpoB* primers; Table 2 contains the complete data) with an additional amplicon of ~700 bp often present. This additional amplicon was present in several of the *B. laterosporus* strains throughout many PCR replicates and would continue to present itself at various annealing temperatures. As expected, all *Paenibacillus* bacteria, the *Brevibacillus* sp. field isolate, and the *Brevibacillus brevis* strain that were tested failed to produce any amplicon, suggesting that these primers are sufficient to correctly identify *B. laterosporus* from any other *Paenibacillus* or *Brevibacillus* strain.

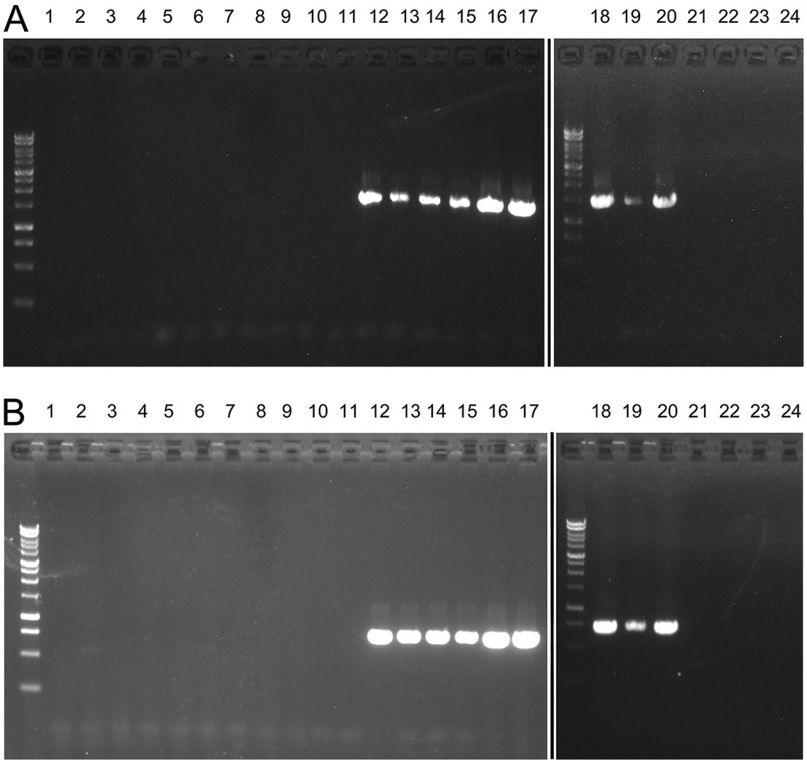

FIG 2 PL *Vagabond* primers discriminate between *P. larvae* and other closely related bacteria. (A and B) *Paenibacillus*-specific primers were used to attempt PCR amplification of a region of the genome found in various control strains as well as field isolates. TAQ (New England BioLabs) PCR was performed according to the manufacturer's instructions with an annealing temperature of 64°C (A) or 60°C (B). Lanes were loaded identically for the two gels as follows (the bacterial template is indicated): 1, *B. laterosporus* 40A1 (ATCC 9141); 2, Br1; 3, Br2; 4, Br3; 5, Br4; 6, Br5; 7, Br6; 8, Br7; 9, Br9; 10, Br14; 11, *B. brevis* (ATCC 8246); 12, *P. larvae* (ATCC 9545); 13, PL309; 14, PL325; 15, PL335; 16, PL337; 17, PL338; 18, PL344; 19, PL345; 20, PL357; 21, *Paenibacillus* sp. (BGSC 35A1); 22, *P. alvei* (BGSC 33A1); 23, *P. polymyxa* (BGSC 25A2); 24, negative control. The black line indicates a separate gel. The ladder included at the far left lane of each gel is a 1-kb DNA ladder (Gold Biotechnology), with the top bright band representing 3 kb and the bottom bright band representing 1 kb.

DISCUSSION

Although 16S rRNA sequencing is a powerful tool for assigning taxonomy of many bacteria, there are several pitfalls when using single-gene analyses to distinguish between bacterial strains (29–38). The presence of both *B. laterosporus* and *P. larvae* in beehives, along with their taxonomic relatedness in the *Paenibacillaceae* family, may facilitate horizontal gene transfer, complicating the identification of bacterial isolates from beehives. These close ecological and evolutionary ties have led to many reported difficulties distinguishing these strains (1–5), as both strains can be isolated together using techniques that are supposed to be *P. larvae* specific. These difficulties may have led to the misclassification of several *B. laterosporus* 16S rRNA sequences in NCBI as *P. larvae*. From our comparisons of 16S rRNA sequences, we conclude that strains DSM 8442, DSM 8443, and BD12OL1-B15 deposited in GenBank are most likely *B. laterosporus*. Based on our results, GenBank currently lists the DSM strains 8442 and 8443 as "unverified." The phage typing system, which has historically been used to address this bacterial isolate identification conundrum, requires a far larger and more diverse collection of *Brevibacillus* and *Paenibacillus* phages than is currently available. However, a multilocus PCR approach can be a powerful, inexpensive, and rapid tool for verifying putative strains.

The use of multigene analysis for the classification of isolated bacteria has been proposed for many years (33). This study highlights this need for multigene analysis for the closely related bacteria *Paenibacillus larvae* and *Brevibacillus laterosporus*, which

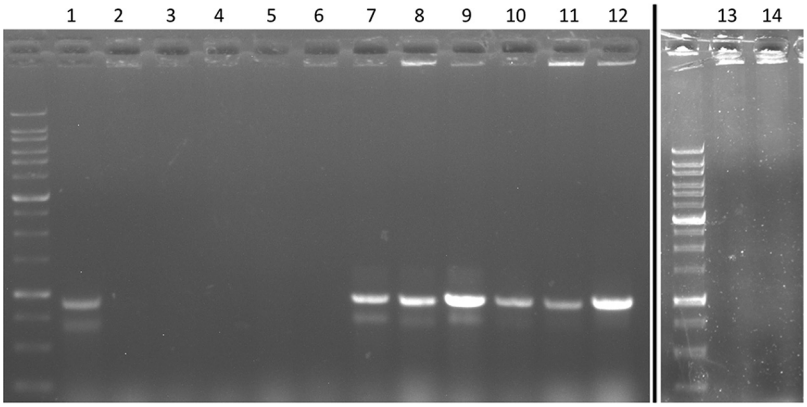

FIG 3 *Brevibacillus*-specific primers for the *rpoB* gene discriminate between *Paenibacillus* and *Brevibacillus* bacteria and verify that field isolates (BL) are *Brevibacillus laterosporus*. *Brevibacillus*-specific primers (BL rpoB) were used to attempt PCR amplification of the *rpoB* gene found in *B. laterosporus* from various control strains as well as field isolates (BL). TAQ (New England BioLabs) PCR was performed according to the manufacturer's instructions with an annealing temperature of 69.1°C. Lanes were loaded as follows (the bacterial template is indicated): 1, *B. laterosporus* 40A1 (ATCC 9141); 2, *B. brevis* (ATCC 8246); 3, *P. larvae* (ATCC 9545); 4, *P. alvei* (BGSC 33A1); 5, *P. polymyxa* (BGSC 25A2); 6, *Paenibacillus* sp. (BGSC 35A1); 7, Br1; 8, Br3; 9, Br4; 10, Br5; 11, Br6; 12, Br12; 13, *Brevibacillus* sp.; 14, PL357. The black line indicates a separate gel. The ladder included in the far-left lane of each gel is a 1-kb Plus DNA ladder (Gold Biotechnology), with the top bright band representing 3 kb and the bottom bright band representing 1 kb.

share an ecological niche. In this work, we demonstrate the utility of three primer sets targeting the *P. larvae Vagabond 1*, *P. larvae Vagabond 2*, and *B. laterosporus rpoB* loci (Fig. 2 and 3), which can be used to distinguish between *Brevibacillus laterosporus* and *Paenibacillus larvae* strains at the species level, and when combined as a multilocus approach can be more accurate than traditional 16S rRNA analysis by itself in identifying these strains at the species level. The use of single-gene analysis should be discouraged due to the close genetic relationship of these genera that share an ecologic niche as exemplified by previous misclassifications. However, with the combined use of these molecular markers (*P. larvae Vagabond 1*, *P. larvae Vagabond 2*, and *B. laterosporus rpoB*), we provide a fast, inexpensive, and reliable method to successfully differentiate these species intimately involved in beehive health beyond the single-locus level.

MATERIALS AND METHODS

Isolation of bacteria from beehives. Bacterial spores were gathered from honey and beehive material as previously described (17). Samples were processed for intended *P. larvae* isolation by isolating spores (19), streaking them on *Paenibacillus larvae* agar (PLA) plates (20), and subjecting them to aerobic incubation at 37°C for 24 to 36 h. Gram-positive isolates that were catalase negative (40) were further streaked and purified on LB (Lennox) agar, archived in 20% glycerol, and stored at −80°C. We originally obtained 11 isolates, which were first presumed to be *P. larvae* and referred to as PL1 through PL10 and PL14. However, of these samples, PL14 was isolated in the same manner as the others but from a diseased larva displaying classical symptoms of American foulbrood. Of these isolates, only PL2 and PL6 were described in the previous studies (17, 18). These 11 isolates are here referred to as *B. laterosporus* strains Br1 through Br10 and Br14, indicative of their identification as *B. laterosporus* rather than as *P. larvae* (41) based on the analyses presented in this study.

Additional samples were gathered from beehives infected with American Foulbrood. From these, *P. larvae* bacterial isolates were obtained from diseased larvae and processed as described previously (20). Notably, isolates were streaked onto MYPGP agar plates (containing 1% Mueller-Hinton broth, 1.5% yeast extract, 0.3% K_2HPO_4, 0.2% glucose, 0.1% sodium pyruvate, and 2% agar) (60) supplemented with nalidixic acid and pipemidic acid and incubated at 35°C in 5% CO_2 for initial spore recovery, followed by further streaking for purification on brain heart infusion (BHI) agar supplemented with glucose and thiamine. Using this updated *P. larvae* isolation protocol, we obtained eight *Paenibacillus larvae* isolates (PL309, PL325, PL335, PL337, PL338, PL344, PL345, and PL357) and one *Brevibacillus* sp. isolate.

Growth of bacterial cultures. Liquid bacterial cultures of *B. laterosporus* were grown in LB broth (Lennox) and incubated overnight while shaking at 200 rpm, 37°C. These media and conditions were also used to grow *B. laterosporus* strain 40A1 (KF597228) (42) from the Bacillus Genetic Stock Center (BGSC;

www.bgsc.org). Liquid cultures of *P. larvae* were grown overnight in BHI broth supplemented with glucose and thiamine (20) while shaking at 120 rpm and 35°C.

Strain identification using PCR and sequencing. Prior to PCR, *P. larvae* and *B. laterosporus* field isolates were streaked out to single colonies. Template DNA for the PCR was extracted by adding part of a colony to 50 μl of distilled or deionized water in a PCR tube and boiling at 100°C for 10 min. TAQ (New England BioLabs) PCR was performed according to the manufacturer's instructions using the primers listed in Table 3. Annealing temperatures for all primers in the PCRs are listed in Table 3. PCR amplicons were electrophoresed using a 1% agarose gel to confirm amplification and verify relative amplicon sizes. Amplicons from reaction mixtures containing 16S rRNA primers or *P. larvae rpoB* primers were sequenced using BigDye sequencing (Life Technologies) and analyzed using BLAST (24–26). The universal 16S rRNA primers were designed as presented by D. J. Lane (43), and the *P. larvae rpoB* and *ftsA* primers were designed by Morrissey et al. (39). All other primers were designed for this study.

The 5′ and 3′ ends of trace files from BigDye sequencing were trimmed for *B. laterosporus* strains Br2, Br4, and Br6 using Geneious version 7.1.7 (44) with an error probability limit of 0.05 but were untrimmed for all other strains as trace files were unavailable (see raw sequence data in the supplemental material). Geneious was used to align sequences obtained from forward and reverse primers to generate a consensus sequence. Sequences of 16S rRNA genes from *B. laterosporus* field isolates were analyzed using online software from the Ribosomal Database Project (RDP) (45) and Greengenes (46) to identify taxonomic relationships with known bacteria. A neighbor-joining phylogenetic tree containing 16S rRNA gene sequences was constructed using MEGA7 (27) with a bootstrap value of 2,000. Any branches with a bootstrapping value below 50% were collapsed. The tree included sequences from the above-referenced field isolates Br1 through Br10 and Br14, as well as several related bacteria and an outgroup bacterium, referenced in Table 1.

***Vagabond* primer design.** *P. larvae Vagabond* primers were designed by using Geneious version 7.1.7 (44) to manually identify genomic regions of various sizes that were conserved among *P. larvae* and no other species. Briefly, we would identify a random region within the genome of interest, ensure that it was found in all other bacteria of that species, and then ensure that this region was not found in any other bacterium of that genus or of other closely related genera. These primers were then experimentally verified to ensure that they amplified the specified region exclusively in *P. larvae*. In addition, we developed a program named UniqSeq for identifying unique sequences in closely related strains to model this design process. To generate primers using this program, all published FASTA genome sequences are collected for the species of interest, along with several published sequences for similar species that could complicate strain identification on field isolates (designated "other"). UniqSeq is then used to produce primer sets unique to our species of interest. UniqSeq is publicly available on GitHub (https://github.com/j-berg/UniqSeq). It is important to realize that this tool, while useful, relies on 100% identity to novel, distinguishing targets for amplification, thus necessitating experimental validation of these primer sets on previously identified strains. Future versions of UniqSeq will account for this shortcoming in order to provide a more useful tool when various levels of sequence conservation or possible off-target effects could complicate amplification of a single target within the species of interest. For the time being, if users opt to use this tool, they should manually check the outputted oligonucleotides' annealing temperatures and any sequence identity to organisms of interest to ensure the accuracy of the oligonucleotides.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01886-18.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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