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Paenibacillus larvae subspecies with dissimilar virulence patterns also group by vegetative growth characteristics and enolase isozyme biochemical properties

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ABSTRACT

Paenibacillus larvae – the causal agent of American foul brood disease in Honey bees – group to different subspecies based upon disease progression and virulence as well as by molecular genotype. Vegetative growth studies reveal that virulence-grouped subspecies arrive at different saturated cell densities. In addition, strains segregating based upon virulence phenotype contain different genotypes in the locus encoding for the key glycolytic enzyme enolase. DNA sequence comparison of enolase loci from 7 *Paenibacillus larvae* strains identified 6 single-nucleotide polymorphisms (SNP) that segregated based on subspecies virulence classification. Only one polymorphism represented a change in amino acid coding (glycine or alanine) at position 331 of the protein. The kinetic properties of two recombinant enolase proteins expressed from enolase alleles isolated from different virulence classed strains (*P. larvae* ATCC 9545 and SAG 10367) yielded a K_m and of 4.2 μM and 1.5 μM and V_{max} of 16.2 μmol min^{-1} mg^{-1} and 10.8 μmol min^{-1} mg^{-1}, respectively. Enolase from *P. larvae* SAG 10367 had a maximum reaction velocity lower than and a specificity constant approximately 1.6 × higher than that of *P. larvae* ATCC 9545.

1. Introduction

Paenibacillus larvae, a gram-positive spore-forming bacterium, is the cause of American foulbrood (AFB) and powdery scale in honey bee larvae (*Apis mellifera* Linnaeus) (White, 1906; Katznelson, 1950). Phenotypic and molecular characteristics place *P. larvae* into two subspecies *P. larvae* subsp. *larvae* (*Pll*) and *P. larvae* subsp. *pulvifaciens (Plp*) (Heyndrickx et al., 1996), A more recent molecular characterization, using ERIC-PCR technology, has generated 4 genotypes (i.e., ERIC I, II, III, IV) (Genersch et al., 2006). ERIC-PCR and subspecies classification do correlate in that ERIC I includes *Pll* strains and ERICs III, and IV contain *Plp* strains. Genotype ERIC II, predominantly contains isolates identified as *Plp* (Genersch et al., 2006) and, for this investigation, *Plp* is considered as containing genotypes ERIC II, III, and IV.

Hitchcock and associates (Hitchcock et al., 1979) demonstrated that disease progression was more rapid for *Plp* than disease progression by *Pll.* In addition, ERIC I genotypes take longer to kill 100% of infected host samples than isolates grouped into ERIC II, III, and IV genotypes (i.e., ~12 days vs ~7 days, respectively) (Ashiralieva and Genersch, 2006; Genersch et al., 2005). An explanation(s) for virulence differences between subspecies, or genotypes, is not known. Strain variations that affect basic metabolic processes are potential candidates and are now accessible through genome sequences.

Enolase is of particular interest since it may influence the pathogenic process by three different routes: as a cell surface protein, as a component of the RNA degradosome or as an enzyme. In gram-positive bacteria enolase is found on cell surfaces and binds to laminin, fibronectin, and collagens (aiding bacterial cell adhesion) and to plasminogen (promoting conversion to plasmin) aiding virulence (Antikainen et al., 2007). Plasmin, a serine protease involved in fibrinolysis and extracellular matrix degradation, can enhance tissue invasion (Saksela and Rifkin, 1998). In *P. larvae* secreted enolase was identified as a potential virulence factor (Antúnez et al., 2010; Antúnez et al., 2011). Whether a plasminogen-binding property is associated

Abbreviations: APSSP2, Advanced Protein Secondary Structure Prediction Server; AFB, American foulbrood; ERIC-PCR, Enterobacterial Repetitive Intergenic Consensus; LB, Luria-Bertani; *Pll*, *P. larvae* subsp. *larvae*; *Plp*, *P. larvae* subsp. *pulvifaciens*; PEP, phosphoenolpyruvate; 2-PGA, 2-phosphoglycerate; PCR, Polymerase chain reaction; SNP, single-nucleotide polymorphisms; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Table 1
Bacterial strains.

Strain	Relevant properties/strain history	Source (reference)
P. larvae subspecies *larvae*; ERIC I genotype		
CT-021709-2	Isolated; Conn., USA (2009)	(Dingman, 2015)
ATCC[a] 9545	NRRL B-2605; Holst 846; (Gordon et al., 1973)	AM Alippi
PL 7	Isolated; Pigüé (Buenos Aires, Argentina)	(Alippi and Aguilar, 1998)
NRRL[b] B-3650	Bailey; Rothamsted Expt. Stat, Harpenden, England; Australia strain (Gordon et al., 1973)	Dingman, 1983
P. larvae subspecies *pulvifaciens*; ERIC II genotype		
SAG 10367[c]	Isolated; honey from Chile	AM Alippi
P. larvae subspecies *pulvifaciens*; ERIC III or IV genotype		
CCM 38	CCUG 7427; NRRL B-1283; Katznelson 113; (Gordon et al., 1973); Ottawa, Canada	(Alippi et al., 2002)
NRRL[b] B-14154	Nakamura received from H. deBarjac; Paris, France	(Alippi et al., 2002)
E. coli		
BL21 (DE3)	*fhuA2 [lon] ompT gal* (λ DE3) [*dcm*] Δ*hsdS* λ DE3 = λ *sBamHIo* Δ*EcoRI-B* int::(*lacI::PlacUV5::T7 gene1) i21* Δ*nin5*	New England Biolabs, Inc., Ipswich, MA
Stellar competent (strain HST08)	F⁻*end*A1 *sup*E44 *thi*-1 *rec*A1 *rel*A1 *gyr*A96 *pho*A φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 Δ(*mrr-hsd*RMS-*mcr*BC) Δ*mcr*A-	Clontech Laboratories, Inc., Mountain View, CA
TOP10	F⁻*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G-	Life Technologies, Carlsbad, CA

[a] ATCC = American Type Culture Collection, Manassas, VA.
[b] NRRL = Northern Regional Research Laboratory, Peoria, IL.
[c] ERIC genotype reclassification; (Dingman, 2015).

with *P. larvae* enolase, and how that property might be associated with virulence is unknown. However, strong evidence exists of a plasminogen-like protein in *A. mellifera* (Grossi et al., 2016). Enolase is also part of the RNA degradosome that indirectly influences both metabolic loci involved in growth (Morita et al., 2004) as well as bacterial virulence by controlling response to oxidative stresses (Weng et al., 2016).

Enolase (EC 4.2.1.11) is a key enzyme in the glycolic pathway facilitating the conversion of 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate (PEP), and is abundant in many organisms (Pancholi, 2001), Enolase activity may serve as a rate-limiting growth factor for *P. larvae* and growth rate might influence speeds of lethality. This investigation examines vegetative growth characteristics and enolase genotypes in different *P. larvae* virulence segregated subspecies. In addition, this research determines the kinetics of two enolase forms.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Paenibacillus larvae and *Escherichia coli* strains used in this study are listed in Table 1. *P. larvae* was grown on MYPGP agar plates or broth (Dingman and Stahly, 1983) and *Escherichia coli* on or in Luria-Bertani (LB) media at 37 °C. Vegetative growth profiles of six independent *P. larvae* strains were inoculated with 1/50 overnight culture into 0.5 mL MYPGP, grown at 37 °C with an orbital shake of 205 CPM in CytoOne 48-well flat bottom sterile microtiter plates (USA Scientific, Inc., Ocala, FL) and optical density (590 nm) was measured every 20 min over 17 h using a Synergy H1 microplate reader (BioTek Instruments, Inc., Winooski, VT) running Gen5 Data Analysis Software ver. 2.07. Turbidity data were analyzed using Microsoft Excel.

2.2. DNA extraction, PCR amplification and sequence determination

Bacterial DNA was isolated using the QIAamp Tissue kit (Qiagen Inc., Santa Clarita, CA). PCR amplifications of enolase genes from *P. larvae* strains used the Phusion High-Fidelity PCR kit (New England BioLabs, Ipswich, MA) according to manufacturer's specifications and enolase primer combinations; Enolase F1 5′ GCGCTGCAGCAAATCTTTAG 3′ & R1 5′ CTCAATCGCAGAGATGATCG 3′ and Enolase F2 5′ TTCATGATTCTGCCTGTTGG 3′ & R2 5′ CCATATTCAGCTCCCCCTCTC 3′, respectively. Resulting DNA products were purified using Qiaquick PCR purification kit (Qiagen Inc., Santa Clarita, CA), and sent to the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT). DNA sequences for the7 *P. larvae* strains enolase genes are listed in NCBI GenBank as accessions KX897947 – KX897953.

2.3. Plasmid construction

Enolase coding regions were individually amplified from *P. larvae* strains ATCC 9545 (*Pll*) and SAG 10367 (*Plp*) using primers PlenoA 5′ CGCGCGGCAGCCATATGACTATTATCTCAGACGTTTACGCC 3′, and PlenoB 5′ GTTAGCAGCCGGATCCTTATTTAAACTTTTTCAGGTTGT 3′ and CloneAmp HiFi PCR Premix (Clontech Laboratories, Inc., Mountain View, CA). PlenoA and PlenoB were designed as fusion primers for in-frame cloning of the *P. larvae* enolase coding region into vector pET-15b. Resulting PCR - products were purified, cut with restriction endonucleasese *Nde*I and *Bam*HI, ligated to *Nde*I and *Bam*HI cut vector DNA using either In-Fusion HD Cloning Plus kit (Clontech Laboratories, Inc., Mountain View, CA) or the standard T4 DNA ligase cloning procedure (Sambrook et al., 1989) then transformed into *E.coli* Stellar cells (Clontech Laboratories, Inc., Mountain View, CA) or TOP10 cells (Invitrogen, Carlsbad, CA). DNA sequence analysis verified the integrity of resulting plasmids pBWL9 (pET-15b + SAG 10367 enolase) and pBWL55 (pET-15b + ATCC 9545 enolase).

2.4. Purification of recombinant P. larvae enolase

Plasmids pBWL9 and pBWL55 were transformed into *E.coli* strain BL21 (DE3) and recombinant enzyme expressed by autoinduction and purified as in Studier (Studier, 2005). Recombinant protein was enriched by passage through nickel columns (Novagen His-Bind Quick 900, EMD Biosciences, Inc., Madison, WI), and dialyzed using 1 L of 20 mM Tris-HCl, 75 mM KCl, and 2 mM $MgSO_4$; pH 7. Molecular weight of recombinant protein was determined using SDS-Polyacrylamide Gel Electrophoresis and molecular weight standards (Precision Plus Protein unstained standards, Bio-Rad Laboratories, Inc., Hercules, CA). Protein concentration was determined by spectrophotometric measurement at A_{280} as determined using ProtParam (ExPASy; http://web.expasy.org/protparam/).

2.5. Enolase biochemistry

Enolase activity assays were performed at 25 °C in 100 μL reaction

buffer (20 mM Tris-HCl, 75 mM KCl, 2 mM $MgSO_4$; pH 7.0) containing 5 mM 2-PGA as substrate. Resulting PEP accumulation was measured at A_{240} with a Hitachi U-4100 spectrophotomer. Enzyme reaction velocities were determined as in Lee et al. (2006). Enzyme kinetics (i.e., K_m and V_{max}) were determined from differing initial reaction velocities as produced by varying 2-PGA concentration (0, 0.3, 0.6, 0.8, 1.25, 2.5, 5 mM) at a constant enzyme concentration. Enolase velocity (μmol · min^{-1} mg^{-1}) was determined from spectrophotometric data using the Beer-Lambert Law and a molar attenuation coefficient of 1.4×10^{-3} M^{-1} cm^{-1} at pH 7.0 and A_{240} for PEP (Yamamoto et al., 2012). Each assay was performed in triplicate and K_m and V_{max} were calculated by nonlinear regression of Michaelis-Menten plots using Microsoft Excel (ver. 15.0.4859.1000) and Solver.

3. Results

3.1. Growth profiles of P. larvae subspecies

Six *P. larvae* strains were used for vegetative growth studies (Table 1). Strains ATCC 9545, PL7 and NRRL B-3650 belong to the *Pll* group and carry the ERIC I genotype. The remaining three strains belong to *Plp group* and display either the ERIC II genotype (SAG 10367), or ERIC III & IV genotypes (CCM 38 and NRRL B-14154). Fig. 1 shows that, except for strain PL 7, the observed rate of bacterial growth during early exponential growth phase (≤ 300 min) was not significantly different for the strains tested. However, it was clear that for the *Pll* strains [slow disease progression strains] a decelerating growth rate in late exponential growth phase and a lower cell density at stationary phase (i.e., maximum level of turbidity) were observed compared to *Plp* strains [rapid disease progression strains] (Hitchcock et al., 1979).

3.2. Enolase sequence comparisons correlate with P. larvae subspecies

Comparison of seven enolase gene sequences generated in this investigation and 3 reference sequence listed in GenBank found 6 single nucleotide polymorphisms (SNPs) (Table 2). Three distinct enolase alleles segregate with ERIC genotypes. One enolase allele (sharing six SNPs) is common to 6 *Pll* strains belonging to ERIC I. A second enolase allele matches with DSM 25430 in ERIC II sharing 5 of 6 SNP with ERIC I strains. The third allele segregates with ERIC II, III and IV strains. Three *Plp* strains (in ERIC II, III, and IV) share one allele and six *Pll* strains share another allele. One *Plp* strain (DSM 25430) displays the allele sharing 5 of 6 SNP bases with *Pll* strains. In fact, strain DSM 25430 was originally identified as *Pll* strain 04-309 (Genersch et al., 2005; Poppinga et al., 2012; Djukic et al., 2014). Because strain DSM 25430 is classified as ERIC II (predominantly containing *Plp* strains; (Genersch et al., 2006)), it is uncertain why the enolase gene sequence more closely related to the *Pll* enolase DNA sequence than to the *Plp* enolase sequence.

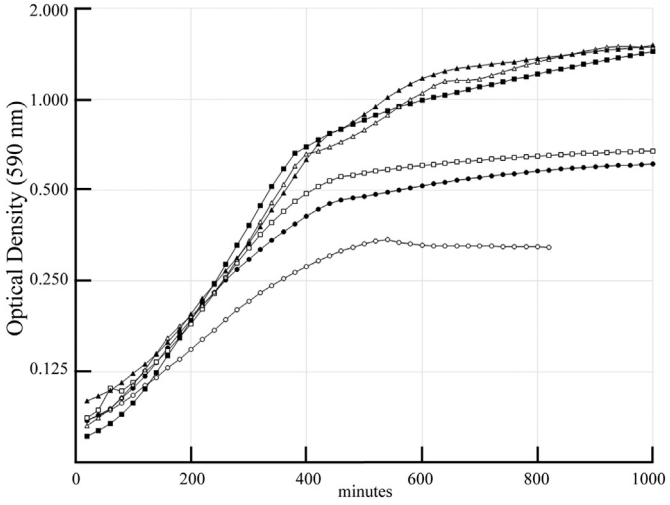

Fig. 1. Growth profiles of *P. larvae* subsp. *larvae* and *P. larvae* subsp. *pulvifaciens* strains in MYPGP broth at 37 with aeration *Paenibacillus larvae* subsp. *larvae* strains included PL 7 (○), ATCC 9545 (●), and NRRL B-3650 (□). *Paenibacillus larvae* subsp. *pulvifaciens* strains included SAG 10367 (▲), CCM 38 (■), and NRRL B-14154 (Δ).

Table 2
P. larvae Enolase nucleotide polymorphism.

Base pair number[a]		27	108	567	804	978	992
Strain	NCBI GenBank						
Pll[b]; ERIC I							
NRRL B-3650	KX897950	C	T	T	T	T	C
BRL-230010	AARF01000086	C	T	T	T	T	C
DSM 25719	ADFW01000001	C	T	T	T	T	C
CT-021709-2	KX897947	C	T	T	T	T	C
ATCC 9545	KX897948	C	T	T	T	T	C
PL 7	KX897949	C	T	T	T	T	C
Plp[c]; ERIC II							
DSM 25430[d]	CP003355	C	T	G	T	T	C
SAG 10367	KX897951	T	C	G	C	C	G
Plp; ERIC III and IV							
CCM 38	KX897952	T	C	G	C	C	G
NRRL B-14154	KX897953	T	C	G	C	C	G

[a] Base pair numbering started at A in first codon of enolase coding region (i.e., ATG).
[b] *Paenibacillus larvae* subsp. *larvae*.
[c] *Paenibacillus larvae* subsp. *pulvifaciens*.
[d] Identified as *Paenibacillus larvae* subsp. *larvae* with an ERIC II genotype.

Only one SNP (at DNA position 992) resulted in an amino acid variation of alanine or glycine at amino acid position 331 (Table 3). The remaining five SNPs represented silent changes. Using APSSP2 (Advanced Protein Secondary Structure Prediction Server; (Raghava, 2000);www.imtech.res.in/raghava/apssp2/), this amino-acid difference was determined to occur between a α-helix and β-sheet in the secondary structure of the protein. The change was also within 4 amino acids of one of the three conserved amino acids (i.e., 164E, 242D, and 335L) reported to comprise the enzymatic active site (Feng et al., 2009). The amino acid polymorphism maintained segregation between the 3 *Plp* strains and the *Pll* (ERIC I) strains. Strain DSM 25430 (ERIC II), as for the DNA sequence, matched *Pll* strains. Comparison of this amino acid location to that of other *Paenibacillus* species identified glycine (present in the *Plp* enolase) as predominant at this location in the protein (Table 3).

3.3. Recombinant enolase purification and activity

Recombinant *P. larvae* enolases were enriched from the *E. coli* BL21 (DE) strains containing plasmids pBWL9 (strain SAG 10367; *Plp*) and pBWL55 (ATCC 9545; *Pll*). SDS-PAGE analysis determined that the recombinant enolases proteins migrated slightly below 50 kDa (Fig. 2, lanes 4 and 7), comparable to the 46 kDa size for enolase in *P. larvae* (Antúnez et al., 2010). The purified enolase isomers from *E. coli* strains containing pBWL9 and pBWL55 had concentrations of 3.9 mg/mL and 3.4 mg/mL, respectively.

To confirm that the purified proteins were active isomer enolases, linked with strains SAG 10367 and ATCC 9545 (*Plp* and *Pll*, respectively), enzymatic assays for enolase were performed using increasing amounts of purified protein (1, 2.5, 5 μL), while keeping substrate concentration constant (5 mM 2-PGA) in the reaction mixture (100 μL). Each purified recombinant protein showed increased enolase activity (i.e., conversion of 2-PGA to PEP) with increased volume (data not presented). The lag-log-plateau trend characteristic of an enzymatic reaction was observed within the first 2 min of the reaction, further confirming the purified proteins to be enzymes (data not presented).

Table 3
Enolase amino acid sequence polymorphism.

Bacterial species and strain	NCBI GenBank	Amino acid sequence*
		321 331
P. larvae ssp. *larvae* (ERIC I)		| |
BRL -230010	IC56_RS00925	-RLSTGIEKGI **A**NSILVKVNQ-
DSM 25719	ETK29332	-RLSTGIEKGI **A**NSILVKVNQ-
NRRL B -3650	KX897950	-RLSTGIEKGI **A**NSILVKVNQ-
CT -0217092	KX897947	-RLSTGIEKGI **A**NSILVKVNQ-
ATCC 9545	KX897948	-RLSTGIEKGI **A**NSILVKVNQ-
PL 7	KX897949	-RLSTGIEKGI **A**NSILVKVNQ-
P. larvae ssp. *pulvifaciens* (ERIC II)		
DSM 25430	AHD04128	-RLSTGIEKGI **A**NSILVKVNQ-
SAG 10367	KX897951	-RLSTGIEKGI GNSILVKVNQ-
P. larvae ssp. *pulvifaciens* (ERIC III & IV)		
CCM 38	KX897952	-RLSTGIEKGI GNSILVKVNQ-
NRRL B -14154	KX897953	-RLSTGIEKGI GNSILVKVNQ-
Paenibacillus alvei TS -15	EPY08730	-RLAQGIEKGI GNSILVKVNQ-
Paenibacillus amylolyticus Heshi-A3	GAS85392	-RLGRGIDEGI GNSILIKVNQ-
Paenibacillus curdlanolyticus YK9	EFM09548	-RLKDGIDQGV GNSILVKVNQ-
Paenibacillus darwinianus MB1	EXX88429	-RLSTGIEKGI GNSILVKVNQ-
Paenibacillus dendritiformis C454	EHQ59810	-RLATGIEKGI GNSILIKVNQ-
Paenibacillus lactis 154	EHB48606	-RLERGIKEGI GNSILIKVNQ-
Paenibacillus polymyxa M1	CCC83111	-RLAKGIDENI GNSILIKVNQ-
Paenibacillus popilliae ATCC14706	GAC41219	-RLATGIEKGI GNSILIKVNQ-
Paenibacillus sp. JDR -2	ACS98861	-RLSDGIEKGV GNSILVKVNQ-
Brevibacillus laterosporus DSM 25	NZ_KB894287	-RLITGIEKAT **A**NSILVKVNQ -
Bacillus subtilis ssp. *spizizenii* W23	ADM39365	-KLAEGIKNGV GNSILIKVNQ-
Bacillus amyloliquefaciens DSM 7	CBI44378	-KLAEGIKNGV GNSILIKVNQ-
Bacillus cereus FRI -35	AFQ09850	-KLAEGIEKGI **S**NSILIKVNQ-
Borrelia burgdorferi B31	AAC66719	-FLKKGIEMGV **A**NSILIKVNQ-
Streptococcus suis 05ZYH33	FJ895346	-YLARGIKEGA **A**NSILIKVNQ-

* Numbering based on *P. larvae* enolase amino acid sequence

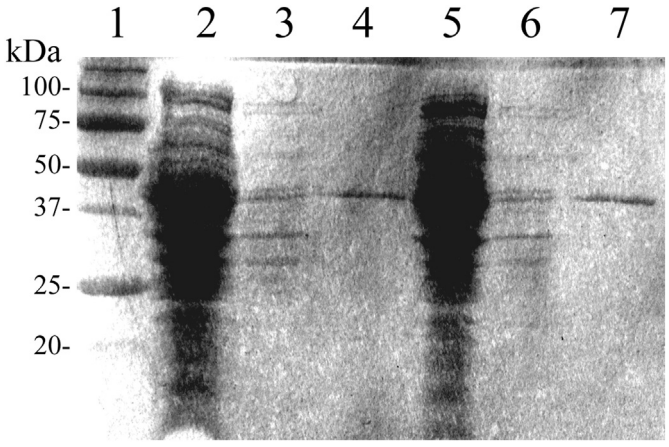

Fig. 2. SDS-PAGE banding patterns of extracted protein purification of His-tagged recombinant *P. larvae* enolase Lanes are; 1) Bio-Rad protein weight standard, 2) *E. coli* pBWL9 (*P. larvae* SAG 10367) cleared lysate, 3) *E. coli* pBWL9 (*P. larvae* SAG 10367) column washate, 4) *E. coli* pBWL9 (*P. larvae* SAG 10367) column eluent, 5) *E. coli* pBWL55 (*P. larvae* ATCC 9545) cleared lysate, 6) *E. coli* pBWL55 (*P. larvae* ATCC 9545) column washate, 7) *E. coli* pBWL55 (*P. larvae* ATCC 9545) column eluent.

3.4. Enolase enzymatic kinetics differ by P. larvae subspecies

Prior to measuring enzyme kinetics for the recombinant enolases, two control assays were performed. To ascertain that variability of substrate concentration in the reaction assay was the only variable affecting enzymatic activity measurements, the assay was performed in the absence of purified recombinant enolase. Lack of enolase resulted in no observed reaction (i.e., spectrophotometric change at A_{240}) for the various 2-PGA concentrations used (data not presented). Also, using increasing volumes (1–7 μL) of nickel-column purified eluent from *E. coli* BL21 (DE3) that lacked plasmids pBWL9 or pBWL 55 (i.e., recombinant enolase) demonstrated no increase in enzymatic activity as measured spectrophotometrically at 240 nm (data not presented). All assays were performed at 25 °C.

Fig. 3 shows results of enzymatic activity assays for the two recombinant *P. larvae* enolases based on varying 2-PGA concentration and a fixed amount of recombinant enzyme. Assays for both recombinant enolases exhibited classic hyperbolic relationships comparing initial enzymatic velocity to substrate concentration, fitting Michaelis-Menten kinetics. Through non-linear regression, enolase from ATCC 9545 was calculated to have a K_m of 4.2 μM for 2-PGA and V_{max} of 16.2 μmol min^{-1} mg^{-1} (r^2 = 0.98). Enolase from strain SAG 10367 had a K_m of 1.5 μM for 2-PGA and V_{max} of 10.8 μmol min^{-1} mg^{-1} (r^2 = 0.85). Based on use of 2.5 μL of enzyme stock (8.5 μg and 9.8 μg of ATCC 9545

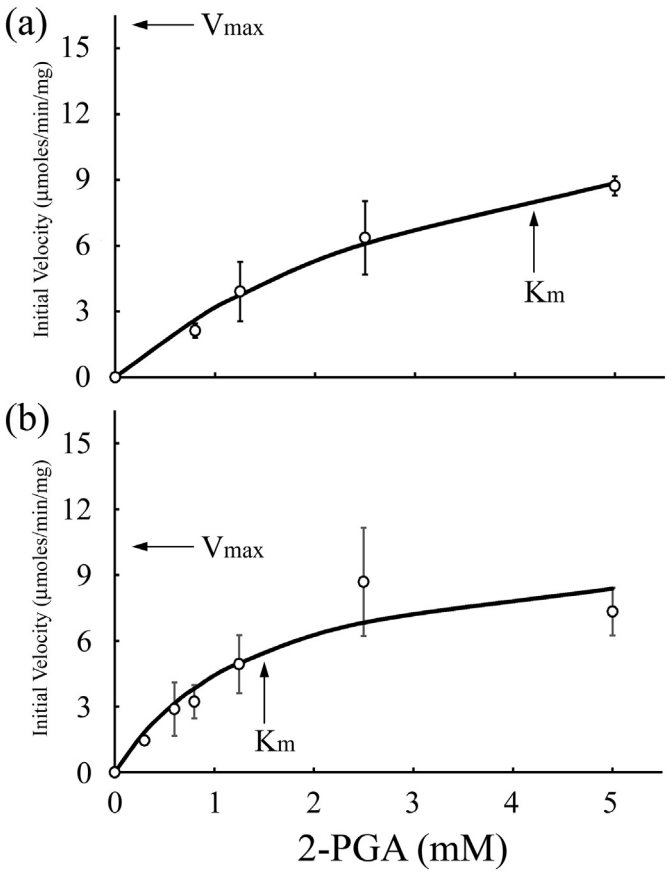

Fig. 3. Enzymatic kinetic parameters showing V_{max} and K_m determined using initial velocity for different 2-PGA concentrations at a fixed amount of recombinant enolase Recombinant enolase produced from the enolase coding region of (a) *P. larvae* ATCC 9545 and (b) *P. larvae* SAG 10367 was used in the reactions.

and SAG 10367 recombinant enolase, respectively) in the reaction mixture, molecular weights of 50,380 Da and 50,366 Da for ATCC 9545 and SAG 10367 recombinant enolases, respectively, and that most bacterial enolases are dimeric (Antikainen et al., 2007), the turnover rates (k_{cat}) were calculated as 812 s^{-1} and 469 s^{-1} for ATCC 9545 and SAG 10367 recombinant enolase, respectively. The enzymatic efficiency (i.e., specificity constant) of recombinant *P. larvae* strain SAG 10367 (*Plp*, ERIC II) enolase for 2-PGA was determined as approximately 1.6 × that of recombinant *P. larvae* ATCC 9545 (*Pll*, ERIC I) enolase. Because variability of the enolase amino acid sequences in the strains investigated was only at a single site, and this variability separated along subspecies, it is concluded that most *P. larvae* strains identified as *Plp* contain an enolase with an enzymatic efficiency for 2-PGA that is 1.6 × higher than enolase in strains identified as *Pll*.

4. Discussion

Genetic differences between *P. larvae* subspecies determine virulence phenotypes (fast or slow progression) (Hitchcock et al., 1979), as well as the disparity in cell density at the stationary stage of vegetative growth (Fig. 1). It is as yet unclear if the growth and virulence characters are influences by the same or overlapping loci. Three reasons support the contention that the enolase gene is a candidate locus that influences both virulence and metabolism. First, enolase is a key metabolic enzyme involved in the conversion of 2-PGA to PEP during cellular glycolysis, and as such is directly involved in vegetative cell growth. Second, enolase is part of the RNA degradosome that indirectly influences both metabolic loci involved in growth (Morita et al., 2004) as well as bacterial virulence by controlling response to oxidative stresses (Weng et al., 2016). Third, enolase has a direct role in pathogenicity by acting as a cell surface binding protein (Genersch et al., 2005; Grossi et al., 2016) and is reported to be a virulence factor in *P. larvae* (Antúnez et al., 2011).

This investigation establishes that there is a genetic difference in the enolase loci that correlates with both vegetative growth and virulence phenotypes. DNA sequence analysis of enolase loci from seven *P. larvae* strains reveals a single SNP that alters the protein sequence at position 331 (Table 3). These two enzyme isoforms segregate along subspecies classification and within ERIC genotypes, except for ERIC II. It is possible that one of the ERIC II strains was misclassified. Interpretation of ERIC classification can be problematic between laboratories (Tyler et al., 1997; Johnson and Clabots, 2000; Di Pinto et al., 2011). Strain SAG 10367, originally identified as ERIC III (Dingman, 2015), has a banding pattern that best matches the ERIC-PCR electrophoretic banding pattern presented by ERIC II and has been reclassified (Genersch et al., 2006). The ERIC II genotype is problematic and may contain isolates that are intermediates to *Pll* and *Plp* (Genersch et al., 2006). Further, differences could be due to use of strains grouped within the same genotype that most likely have been geographically segregated for an extensive time. Strain SAG 10367 was originally isolated in South America, while strain DSM 25430 was an isolate from Germany.

The single amino acid change at position 331 in the *P. larvae* enolase produced a different affinity and turnover rate for 2-PGA. The enolase found within strains of *Plp* demonstrated an affinity for substrate (i.e., K_m) 2.8 times, and an enzymatic efficiency 1.6 times, that of enolase present in *Pll* (ERIC I) strains. The replacement of glycine by alanine within a α-helix structure of a protein is known to stabilize that structure (Scott et al., 2007) in that alanine provides less flexibility in the protein (Yan and Sun, 1997). Differences in rigidity can effect enzymatic activity by influencing conformational changes or producing structural stress at the reactive site (Yan and Sun, 1997). Although the change in *P. larvae* enolase occurred between a α-helix and β-sheet structure, it was within 4 amino-acids of one of the three conserved amino-acids defined as forming the active site of enolase (Feng et al., 2009). Lowered flexibility at this location, due to the presence of alanine, may explain the lower enzymatic affinity for substrate observed in the *Pll* strains. This “rigidity,” by stabilizing the reactive site, may also explain the increased turnover rate observed for the *Pll* strains. What advantage/disadvantage this amino-acid change in enolase might have provided for the Pll strains is unknown considering that all other paenibacilli examined (Table 3) contained glycine at position 331.

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A change in enolase flexibility may also influence plasminogen binding properties or its interaction in and function of the RNA degradosome. The change in amino acid at position 331 is outside the presumed binding regions for plasminogen (nucleotides 250–255 and C terminus) and magnesium (enzyme cofactor; 241D, 285E, and 312D) (Feng et al., 2009). Future investigations are needed to determine any effect of protein sequence on plasminogen binding or RNA degradosome properties.

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Conflict of interest

The authors attest that there is no conflict of interest associated with this research.

References

Alippi, A.M., Aguilar, O.M., 1998. Characterization of isolates of *Paenibacillus larvae* subsp. *larvae* from diverse geographical origin by the polymerase chain reaction and BOX primers. J. Invertebr. Pathol. 72, 21–27.

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

Antikainen, J., Kuparinen, V., Lähteenmäki, K., Korhonen, T.K., 2007. Enolases from Gram-positive bacterial pathogens and commensal lactobacilli share functional similarity in virulence-associated traits. FEMS Immunol. Med. Microbiol. 51, 526–534.

Antúnez, K., Anido, M., Evans, J.D., Zunino, P., 2010. Secreted and immunogenic proteins produced by the honeybee bacterial pathogen, *Paenibacillus larvae*. Vet. Microbiol. 141, 385–389.

Antúnez, K., Anido, M., Arredondo, D., Evans, J.D., et al., 2011. *Paenibacillus larvae* enolase as a virulence factor in honeybee larvae infection. Vet. Microbiol. 147, 83–89.

Ashiralieva, A., Genersch, E., 2006. Reclassification, genotypes and virulence of *Paenibacillus larvae*, the etiological agent of American foulbrood in honeybees - a review. Apidologie 37, 411–420.

Di Pinto, A., Novello, L., Terio, V., Tantillo, G., 2011. ERIC-PCR genotyping of *Paenibacillus larvae* in southern Italian honey and brood combs. Curr. Microbiol. 63, 416–419.

Dingman, D.W., 2015. Comparative analysis of *Paenibacillus larvae* genotypes isolated in Connecticut. Arch. Microbiol. 197, 789–795.

Dingman, D.W., Stahly, D.P., 1983. Medium promoting sporulation of *Bacillus larvae* and metabolism of medium components. Appl. Environ. Microbiol. 46, 860–869.

Djukic, M., Brzuszkiewicz, E., Fünfhaus, A., Voss, J., et al., 2014. How to kill the honey bee larva: genomic potential and virulence mechanisms of *Paenibacillus larvae*. PLoS One 9, e90914.

Feng, Y., Pan, X., Sun, W., Wang, C., et al., 2009. *Streptococcus suis* enolase functions as a protec7tive antigen displayed on the bacterial cell surface. J. Infect. Dis. 200, 1583–1592.

Genersch, E., Ashiralieva, A., Fries, I., 2005. Strain-and genotype-specific differences in virulence of *Paenibacillus larvae* subsp. *larvae*, a bacterial pathogen causing American foulbrood disease in honeybees. Appl. Environ. Microbiol. 71, 7551–7555.

Genersch, E., Forsgren, E., Pentikäinen, J., Ashiralieva, A., et al., 2006. Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. Int. J. Syst. Evol. Microbiol. 56, 501–511.

Gordon, R.E., Haynes, W.C., Pang, C.H., 1973. The genus *Bacillus*. In: Agriculture Handbook No. 427. Agricultural Research Service, US Dept Agriculture, Washington DC.

Grossi, G., Grimaldi, A., Cardone, R.A., Monné, M., et al., 2016. Extracellular matrix degradation via enolase/plasminogen interaction: evidence for a mechanism conserved in Metazoa. Biol. Cell. 108, 161–178.

Heyndrickx, M., Vandemeulebroecke, K., Hoste, B., Janssen, P., et al., 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. Evol. Microbiol. 46, 270–279.

Hitchcock, J.D., Stoner, A., Wilson, W.T., Menapace, D.M., 1979. Pathogenicity of *Bacillus pulvifaciens* to honey bee larvae of various ages (Hymenoptera: Apidae). J. Kansas Entomol. Soc. 52, 238–246.

Johnson, J.R., Clabots, C., 2000. Improved repetitive-element PCR fingerprinting of *Salmonella enterica* with the use of extremely elevated annealing temperatures. Clin. Diagn. Lab. Immunol. 7, 258–264.

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

Lee, J., Kang, H., Moon, Y., Cho, D., et al., 2006. Cloning, expression and characterization of an extracellular enolase from *Leuconostoc mesenteriodes*. Microbiol. Lett. 259, 240–248.

Morita, T., Kawamoto, H., Mizota, T., Kiada, T., et al., 2004. Enolase in the RNA degradosome plays a crucial role in the rapid decay of glucose transporter mRNA in the response to phosophosugar stress in *Escherichia coli*. Mol. Micribiol. 54, 1063–1075.

Pancholi, V., 2001. Multi-functional α-enolase: its role in diseases. Cell. Mol. Life Sci. 58, 902–920.

Poppinga, L., Janesch, B., Fünfhaus, A., Sekot, G., et al., 2012. Identification and functional analysis of the S-layer protein SplA of *Paenibacillus larvae*, the causative agent of American Foulbrood of honey bees. PLoS Pathog. e1002716.

Raghava, G.P.S., 2000. Protein secondary structure prediction using nearest neighbor and neural network approach. CASP 4, 75–76.

Saksela, O., Rifkin, D.B., 1998. Cell-associated plasminogen activation: regulation and physiological functions. Ann. Rev. Cell Biol. 4, 93–120.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, Second ed. Cold Spring Harbor Laboratory Press, New York.

Scott, K.A., Alonso, D.O., Sato, S., Fersht, A.R., et al., 2007. Conformational entropy of alanine versus glycine in protein denatured states. Proc. Natl. Acad. Sci. 104, 2661–2666.

Studier, F.W., 2005. Protein purification by auto-induction in high-density shaking cultures. Protein Expr. Purif. 41, 207–234.

Tyler, T.D., Wang, G., Tyler, S.D., Johnson, W.M., 1997. Factors affecting reliability and reproducibility of amplification-based DNA fingerprinting of representative bacterial pathogens. J. Clin. Microbiol. 35, 339–346.

Weng, Y., Chen, F., Lio, Y., Zhao, Q., et al., 2016. Pseudomonas aeruginosa enolase influences bacterial tolerance to oxidative stresses and virulence. Front. Microbiol. 7. http://dx.doi.org/10.3389/fmicb.2016.01999.

White, G.F., 1906. The Bacteria of the Apiary, with Special Reference to Bee Diseases, Bureau of Entomology Technical Series no 14. US Depart Agricult, Washington DC.

Yamamoto, S., Gunji, W., Suzuki, H., Toda, H., et al., 2012. Overexpression of genes encoding glycolytic enzymes in *Corynebacterium glutamicum* enhances glucose metabolism and alanine production under oxygen deprivation conditions. Appl. Environ. Microbiol. 78, 4447–4457.

Yan, B.X., Sun, Y.Q., 1997. Glycine residues provide flexibility for enzyme active sites. J. Biol. Chem. 272, 3190–3194.