Agri Gene 6 (2017) 31–36

Contents lists available at ScienceDirect

Agri Gene

journal homepage: www.elsevier.com/locate/aggene

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

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ARTICLE INFO

Keywords:
American foulbrood
Enolase
Paenibacillus larvae

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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http://dx.doi.org/10.1016/j.aggene.2017.09.002
Received 10 March 2017; Received in revised form 2 June 2017; Accepted 13 September 2017
Available online 18 September 2017

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*Eco*RI-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*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) 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*lac*ZΔM15 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