Comparison of *in vitro* methods for the production of *Paenibacillus larvae* endospores

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Abstract

Paenibacillus larvae endospores are the infectious particles of the honey bee brood disease, American Foulbrood. We demonstrate that our previously published protocol (Alvarado et al., 2013) consistently yields higher numbers and purer preparations of *P. larvae* endospores, than previously described protocols, regardless of the strain tested (B-3650, B-3554 or B-3685).

Keywords: *Paenibacillus larvae*, American Foulbrood disease, spore production, honey bee

The Gram-positive, spore-forming bacterium *Paenibacillus larvae* is the causal agent of American Foulbrood disease (White, 1907). Honey bee larvae become infected when contaminated brood food containing infectious spores is fed to larvae by the “nurse” bees, which, like all adult honey bees, are resistant to AFB (Wilson, 1971). The spores of *P. larvae* are highly resilient and can remain viable for at least 35 years (Haseman, 1961). Eradication of *P. larvae* spores from infected hives is commonly achieved by burning the entire hive and all associated equipment (Calderone, 2001; Matheson, 1993), but this highly destructive solution increases the economic losses associated with this disease. Targeted biological approaches that eradicate spores from equipment have significant potential, but the development of such strategies requires large scale and pure preparations of *P. larvae* endospores.

While three procedures for *in vitro* sporulation of *P. larvae* have been commonly used ((Dingman, 1983; Dingman and Stahly, 1983; Genersch et al., 2005) and recently described in (de Graaf et al., 2013)), these methods do not yield large enough numbers of spores of sufficient purity for studies that seek to identify chemical inhibitors and/or germinants of *P. larvae* spores (Akoachere et al., 2007; Alvarez et al., 2010; Howerton et al., 2011; Powell, 1950; Ramirez and Abel-Santos, 2010). Consequently, a new method was developed [“UNLV”; (Alvarado et al., 2013)]. Here, we present a comparison of the UNLV *in vitro* sporulation protocol (Alvarado et al., 2013) with a protocol (described in de Graaf et al., 2013) that has traditionally yielded the highest number of spores (Dingman, The Connecticut Agricultural Experiment Station; personal communication). Notably, in both protocols spores are prepared from solid growth medium.

To account for strain differences, three different *P. larvae* strains were used in this comparative study (Table 1). In the first protocol (de Graaf et al., 2013), spores were harvested from MYPGP agar inoculated with *P. larvae*. Since “sporulation efficiency can decline when high numbers of colonies are present on a plate” (de Graaf et al., 2013), cultures were serially diluted using MYPGP broth to obtain both low (50-200) and high (1,000-5,000) CFU counts per

MYPGP plate prior to spore preparation. This allowed a thorough comparison of this protocol (de Graaf et al., 2013) with the other (Alvarado et al., 2013). After 7 days of incubation, spores were removed from MYPGP plates by gently scraping and washing the agar surface with sterile water three times. The spore suspension was concentrated via centrifugation at 12000 rpm/19800 g at 4 °C for 15 minutes, the supernatant was discarded and the remaining pellet was resuspended in sterile ice-cold water. Additional centrifugation and pellet resuspension steps were repeated four times to clean spores. Spore stocks produced in this manner were stored in sterile distilled water at 4°C.

TABLE 1: Strains of *P. larvae* used.

Species	ERIC classification ^a	Strain number ^b	Isolation
<i>P. larvae</i>	I	NRRL B-3650	Isolated from diseased honey bee, RE Gordon
<i>P. larvae</i>	I	NRRL B-3554	Isolated from diseased honey bee larvae, NRRL
<i>P. larvae</i>	IV	NRRL B-3685 /ATCC-49843	Powdery scale of honey bee larvae, RE Gordon

^aEvaluated at UNLV by Enterobacterial repetitive intergenic consensus sequence-based PCR (Genersch et al., 2006). ^bStrain numbers sourced from the American Tissue Culture Collection (www.atcc.org) are prefixed with ATCC, those from the Agriculture Research Services (ARS) Culture Collection Database Server (nrml.ncaur.usda.gov; formerly known as the Northern Regional Research Laboratories, NRRL) are prefixed with NRRL.

In contrast, the UNLV method utilized Tryptic Soy Agar (TSA) plates (supplied by Becton, Dickinson and Company) inoculated with 200 µl of an exponentially growing culture (about 1.2×10^8 colony forming units, CFUs) that were incubated for 7 days in a 5% CO₂ incubator at 37°C to produce lawns of *P. larvae* (Alvarado et al., 2013). The resulting spore-containing bacterial lawns were collected by flooding plates with ice-cold deionized water. Spores were pelleted by centrifugation at 8000 rpm/8820 g at 4 °C for 5 minutes and resuspended in fresh deionized water. After three washing steps, the spores were separated from their vegetative and partially sporulated forms by centrifugation at 11500 rpm/18200 g at 4 °C for 35 minutes through a 20-

50% HistoDenz™ (Sigma Aldrich) gradient. Spore pellets were washed five times with sterile distilled water and stored at 4°C.

To measure the concentration of viable spores in each spore stock, counts of heat resistant colony forming units were calculated from MYPGP agar plates (Dingman, 1983). Briefly, spore stocks were normalized to an OD₅₈₀ of 0.2, heated at 68°C for 15 minutes, diluted in water and plated onto MYPGP agar. Plates bearing about 30-300 colonies per plate were used to calculate the number of heat-resistant spores in each stock (Dingman, 1983). Assays were performed in triplicate and average CFUs, standard deviations and statistically significant differences ($p < 0.05$) for all pairs of mean heat resistant spore counts per stock were determined using a Student's one-tailed *t* test assuming equal variances.

As expected, spore yields varied from strain to strain regardless of the protocol was used. Consistent with previous findings, yields from B-3554 and B-3650 were higher when spores were harvested from plates with lower CFU density than higher; a trend not seen for B-3685. Importantly, regardless of the strain tested or whether the spores were isolated from either low or high density plates, the UNLV protocol (Alvarado et al., 2013) yielded significantly higher numbers of spores than the established MYPGP plate method (de Graaf et al., 2013) (more than 385-fold higher for strain B-3554, 2.6-fold higher for strain B-3650 and 142-fold higher for strain B-3685; see Figure 1A), allowing us to conclude that the UNLV protocol routinely yields higher numbers of viable spores than the de Graaf protocol, regardless of the *P. larvae* strain used.

The purity of spore stocks was next determined by microscopic analysis. Briefly, 10 µl of each spore stock was smeared onto a glass slide, air dried, and observed after Schaeffer-Fulton staining (Schaeffer and Fulton, 1933). At least 10 images of each spore stock were randomly acquired and three random images were analyzed using image processing software (ImageJ, NIH). The purity of spore preparations viewed in these images was expressed as: (number of

spores/number of spores and vegetative cells) X 100. Statistically significant differences ($p < 0.05$) for all pairs of mean spore preparations or percent purity were determined using a Student's one-tailed t test assuming equal variances.

This analysis revealed that spore stocks prepared from strains B-3554 and B-3685 using the de Graaf protocols consisted of less than 20% spores (Figure 1B). Furthermore, spores

prepared using this protocol, were frequently contaminated with cell debris, although the amount was difficult to quantify by microscopy. For example, even though spore stocks generated from strain B-3650 were found to be 100% pure (did not contain any vegetative cells) cell debris could still be observed by microscopy (Figure 2A). These results indicate that four washes used in the previously reported protocols (de Graaf et al., 2013; Dingman and Stahly, 1983) were not sufficient to remove cell debris from the spore stocks. While it is possible to remove vegetative cells from spore stocks by heating, this approach is not recommended because mucopeptides released from vegetative cells have been shown to trigger spore germination (Shah et al., 2008). In the UNLV protocol, this problem was solved by using a HistoDenz™ density gradient. This approach separated the spores from vegetative cells and cell debris and yielded virtually

pure preparations of spores, regardless of the strain tested (Figure 2B).

In sum, this comparative analysis demonstrates that the UNLV *in vitro* sporulation method is more effective at generating higher numbers of spores with greater purity than the established *in vitro* sporulation protocol (de Graaf et al., 2013; Dingman and Stahly, 1983). The use of a

commercially available rich medium, TSA, facilitated spore production and streamlined media production compared to other protocols (de Graaf et al., 2013; Dingman, 1983; Dingman and Stahly, 1983; Genersch et al., 2005) and the use of a centrifugation density gradient ensured that a pure stock was obtained. Although, we acknowledge that similar results might be obtained if the numbers of plates and tubes were increased while following older protocols (Dingman, 1983; Dingman and Stahly, 1983; Genersch et al., 2005), a differential centrifugation step would still be required to increase the purity of the spores generated. This comparative analysis highlights the strengths and weaknesses of the currently published protocols for the *in vitro* preparation of *P. larvae* spores from solid medium (Alvarado et al., 2013; de Graaf et al., 2013), and as such will help those requiring large scale and pure preparations of *P. larvae* endospores and possibly other bacterial endospores.

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