

Requirements for *In Vitro* Germination of *Paenibacillus larvae* Spores

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Paenibacillus larvae is the causative agent of American foulbrood (AFB), a disease affecting honey bee larvae. First- and second-instar larvae become infected when they ingest food contaminated with *P. larvae* spores. The spores then germinate into vegetative cells that proliferate in the midgut of the honey bee. Although AFB affects honey bees only in the larval stage, *P. larvae* spores can be distributed throughout the hive. Because spore germination is critical for AFB establishment, we analyzed the requirements for *P. larvae* spore germination *in vitro*. We found that *P. larvae* spores germinated only in response to L-tyrosine plus uric acid under physiologic pH and temperature conditions. This suggests that the simultaneous presence of these signals is necessary for spore germination *in vivo*. Furthermore, the germination profiles of environmentally derived spores were identical to those of spores from a biochemically typed strain. Because L-tyrosine and uric acid are the only required germinants *in vitro*, we screened amino acid and purine analogs for their ability to act as antagonists of *P. larvae* spore germination. Indole and phenol, the side chains of tyrosine and tryptophan, strongly inhibited *P. larvae* spore germination. Methylation of the N-1 (but not the C-3) position of indole eliminated its ability to inhibit germination. Identification of the activators and inhibitors of *P. larvae* spore germination provides a basis for developing new tools to control AFB.

American foulbrood (AFB) is a bacterial disease of honey bees that kills the developing larvae (1, 2). *Paenibacillus larvae* spores are the infectious agents for AFB, but it is the vegetative cells that cause disease (3, 4). In 2005, a survey of almond-pollinating bee colonies indicated that 4% of colonies had a significant AFB load (5). Once a beekeeping operation is contaminated, the bacterial spores are not easily removed (6). Although autoclaving and high concentrations of chemical disinfectants effectively kill the spores, these treatments are not viable for the beekeeping industry (7). Traditionally, Terramycin and other antibiotics have been used for the treatment and prevention of AFB. However, antibiotic treatment is ineffective in the spore stage of *P. larvae*, and overuse of the antibiotics leads to resistant strains (8, 9). Presently, the only accepted practice for controlling the spread of AFB is burning both the infected colonies and beekeeping equipment (3, 6).

AFB occurs when first- or second-instar larvae (within 48 h after the egg hatches) ingest food that is contaminated with the *P. larvae* spores (10). Twelve hours after ingestion, *P. larvae* spores germinate and the new vegetative cells start to proliferate inside the larval gut (11). Several days postinfection, extreme bacteremia causes the death of the honey bee larvae (12–14). After the nutrient levels of the honey bees are depleted, *P. larvae* cells stop dividing and then sporulate. As a result, billions of spores are found in the dead remains of each bee larva (15, 16). Within the colony, spores are transmitted by adult bees that eat larval remains (17, 18). *P. larvae* spores are transmitted between colonies through bees that rob honey from neighboring infected colonies and through the use of contaminated beekeeping equipment (19).

Because *P. larvae* spore germination is the first step of infection, controlling spore germination might lead to new approaches to prevent AFB (20). However, little is known about the environmental cues required to trigger *P. larvae* spore germination. In *Clostridia* and *Bacilli* species, spores require sugars, nucleosides, amino acids, and/or inorganic salts to stimulate germination (21). The complexity of germination signals varies and commonly re-

quires several types of germinants (22). We, and others, have also identified molecules that can inhibit spore germination (23–28).

In this work, we tested the ability of metabolites to promote *P. larvae* spore germination. We found that *P. larvae* spores exclusively recognize L-tyrosine and uric acid as cogerminants. We determined the germination 50% effective concentrations (EC₅₀s) of L-tyrosine and uric acid. Because L-tyrosine and uric acid are strong germinants *in vitro*, we screened chemical analogs for their ability to inhibit spore germination. Indole and phenol, the side chains of tryptophan and tyrosine, were thus identified as germination inhibitors. Methylation of the N-1 position of indole inactivates its inhibitory activity. In contrast, methylation of the C-3, C-5, or C-7 position has no effect on the inhibitory potential of indole.

MATERIALS AND METHODS

Materials. Chemicals were purchased from the Sigma-Aldrich Corporation (St. Louis, MO). The dehydrated culture medium was purchased from BD Difco (Franklin Lakes, NJ). *Paenibacillus larvae* subsp. *pulvificiens* strain ATCC 49843 was purchased from the American Tissue Culture Collection (ATCC). Environmental American foulbrood scales (the remains of infected larvae collected from infected hives) were kindly donated by Jay D. Evans at the USDA Bee Research Facility (Beltsville, MD). The environmental strain was identified as a strain of *Paenibacillus larvae* subsp. *larvae* based on its phenotypic characteristics and 16S rRNA analysis (29).

***P. larvae* spore preparation.** *P. larvae* strains were grown on BD tryptic soy agar plates for 7 days in a 5% CO₂ incubator at 37°C. The resulting bacterial lawns were collected by flooding with ice-cold deionized water. Spores were pelleted by centrifugation and resuspended in fresh deionized

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water. After three washing steps, the spores were separated from their vegetative and partially sporulated forms by centrifugation through a 20%-to-50% HistoDenz gradient. The spore pellet was washed five times with water and stored at 4°C (23). Spore preparations were >90% pure as determined by microscopic observation of Schaeffer-Fulton-stained samples (30).

Preparation of germinant solution. Sixteen complex media (Muel-ler-Hinton broth, yeast extract, potassium phosphate, glucose, and pyruvate [MYPGP]; tryptic soy broth [TSB]; brain heart infusion [BHI]; Nutrient; LB; Tris-maleate buffer, yeast extract, glucose, and sodium pyruvate [TMYGP]; NZ amine; NZCYM [NZ amine, NaCl, Bacto yeast extract, Casamino Acids, MgSO₄·7H₂O, adjusted to pH 7.0 with NaOH]; Lactobacillus; SOC [Bacto tryptone, Bacto yeast extract, 5 M NaCl, 1 M KCl, 1 M MgCl₂, 1 M MgSO₄, and 1 M glucose]; Bailey; Clostridium; Michael; Terrific; MD [potassium phosphate (pH 7.5), trisodium citrate, 2% (wt/vol) glucose, ferric ammonium citrate, 0.25% (wt/vol) potassium aspartate, magnesium sulfate, L-tryptophan, and L-phenylalanine]; and Jansen [J] broths) were prepared (31–33). A defined medium was prepared as described previously (34). An artificial worker jelly (AWJ) medium was prepared based on modifications to the published composition of worker jelly (35). For AWJ, the following stock solutions were prepared: 100 mM inosine in 220 mM NaOH, 400 mM for each sugar (fructose, glucose, and arabinose) in water, 30 mM for each of the 20 proteinogenic L-amino acids in 0.36 N HCl, 100 mM uric acid in 220 mM NaOH, and 0.2 mg/ml vitamins (thiamine, riboflavin, pyridoxine, β-alanine, *para*-aminobenzoic acid, nicotinic acid, pantothenic acid, biotin, folic acid, and inositol) in water. To prepare AWJ, inosine, uric acid, sugars, and amino acids were dissolved to a final concentration of 3 mM in 0.1 M sodium phosphate buffer (0.06 mM Na₂HPO₄ and 0.04 mM NaH₂PO₄) and adjusted to pH 7.0. This solution was supplemented with vitamins to a final concentration of 1 μg/ml.

Determination of germinants for *P. larvae* spores. The decrease in optical density (OD) is inversely proportional to spore germination (36). Changes in light diffraction during spore germination were monitored at 580 nm (optical density at 580 nm [OD₅₈₀]) on a BioMate 5 (Thermo Electron Corporation, Waltham, MA) or a Tecan Infinite M200 (Tecan Group, Männedorf, Switzerland) spectrophotometer. Experiments were carried out in 96-well plates (200 μl/well). In preparation for germination assays, *P. larvae* spore suspensions were washed three times with water. Spores were then heat-activated at 70°C for 30 min. The heat-activated spores were allowed to reach room temperature and were transferred to 0.1 M sodium phosphate buffer (pH 7.0) to an approximate OD₅₈₀ of 0.70. Spores were monitored for autogermination for 30 min. Germination experiments were carried out with spores that did not autogerminate. Putative germinants were added individually or in various combinations to a final concentration of 3 mM. Experiments were performed in triplicate with at least two different spore preparations. After germinant addition, the OD₅₈₀ of the spore suspension was measured every minute for an hour. Relative OD values were derived by dividing each OD₅₈₀ reading by the initial OD₅₈₀. Spore germination rates (*v*) were calculated from the initial linear decrease in relative OD (23). Germination rates were set to 100% for *P. larvae* spores that had the highest germination rate in a particular assay. Germination rates for other conditions were divided by the maximum germination rate for that assay and are reported as percent germination (23). Standard deviations were calculated from at least six independent measurements and were typically below 10%. Spore germination was confirmed in selected samples by microscopic observation of Schaeffer-Fulton-stained aliquots (30).

Effect of temperature and pH on *P. larvae* germination. For temperature experiments, *P. larvae* spores were germinated in 3 mM L-tyrosine and 3 mM uric acid. Germination rates were determined as described above, except that the germination temperature varied between 25 and 42°C. The germination rate was set to 100% for the spores that were germinated at 42°C. Germination rates for other conditions were divided by the maximum germination rate at 42°C and are

reported as percent germination. Germination rate differences were analyzed using analysis of variance (ANOVA) followed by a Tukey-Kramer procedure (SigmaPlot v.9).

For pH experiments, *P. larvae* spores were resuspended in 0.1 M sodium phosphate, potassium-sodium phosphate, or citrate phosphate buffer. The pH of the buffers was adjusted between 3.0 and 9.0. Spores were germinated in the presence of 3 mM L-tyrosine and 3 mM uric acid. Germination rates were determined as described above. Germination rate was set to 100% for spores germinated at pH 7.0. Germination rates for other conditions were divided by the maximum germination rate at pH 7 and are reported as percent germination. As discussed above, germination rate differences were analyzed using ANOVA followed by a Tukey-Kramer procedure (SigmaPlot v.9).

Activation of *P. larvae* spore germination by L-tyrosine and uric acid. *P. larvae* spore germination was tested using different combinations of L-tyrosine and uric acid. For L-tyrosine titrations, spores were exposed to various concentrations of L-tyrosine and a constant concentration of 3 mM uric acid. For uric acid titrations, spores were exposed to various concentrations of uric acid and a constant concentration of 3 mM L-tyrosine. Germination rates were determined as described above. The germination rate was set to 100% for *P. larvae* spores germinated in the presence of 3 mM L-tyrosine–3 mM uric acid. Germination rates for other conditions were divided by the maximum germination rate obtained with 3 mM L-tyrosine–3 mM uric acid and are reported as percent germination. Percent germination was plotted against compound concentrations. The resulting sigmoidal curves were fitted using the four-parameter logistic function of the SigmaPlot v.9 software to calculate EC₅₀s (for enhancers of spore germination). EC₅₀ is defined as the concentration of a germinant required to increase the germination rate to 50% of its maximal value (37, 38).

Agonists of *P. larvae* spore germination. To test for possible agonists of *P. larvae* spore germination, spores were individually supplemented with 3 mM purine analog and 3 mM L-tyrosine. Separately, *P. larvae* spores were incubated with 3 mM amino acid analog and 3 mM uric acid. Spore germination was monitored as described above. Germination rates for other conditions were divided by the maximum germination rate obtained with 3 mM L-tyrosine–3 mM uric acid and are reported as percent germination.

Antagonists of *P. larvae* spore germination. To test for possible antagonists of *P. larvae* spore germination, spores were individually supplemented with 3 mM purine analog or 3 mM amino acid analog. Spore suspensions were incubated for 15 min at room temperature while monitoring the OD₅₈₀s. If no germination was detected, L-tyrosine and uric acid were added to final concentrations of 3 mM, and germination was monitored as described above. Germination rates for other conditions were divided by the uninhibited maximum germination rate obtained with 3 mM L-tyrosine–3 mM uric acid and are reported as percent germination.

Inhibition of *P. larvae* spore germination by indole and phenol. *P. larvae* spores were individually incubated with various concentrations of indole, phenol, 1-*N*-methylindole, 3-methylindole, 5-methylindole, or 7-methylindole. After 15 min incubation, the spores were treated with 3 mM L-tyrosine–3 mM uric acid. Germination rate was set to 100% for *P. larvae* spores germinated in the absence of inhibitor. Germination rates for other conditions were divided by the uninhibited maximum germination rate obtained with 3 mM L-tyrosine–3 mM uric acid and are reported as percent germination. Percent germination was plotted against the inhibitor concentrations. The resulting sigmoidal curves were fitted using the four-parameter logistic function in SigmaPlot v.9 to calculate the germination 50% inhibitory concentrations (IC₅₀s) (23, 37).

RESULTS

We were unable to detect significant *P. larvae* spore germination in any of the 16 different complex media tested, even after 24 h incubation. By comparison, spores of *Bacillus anthracis* and *Bacil-*

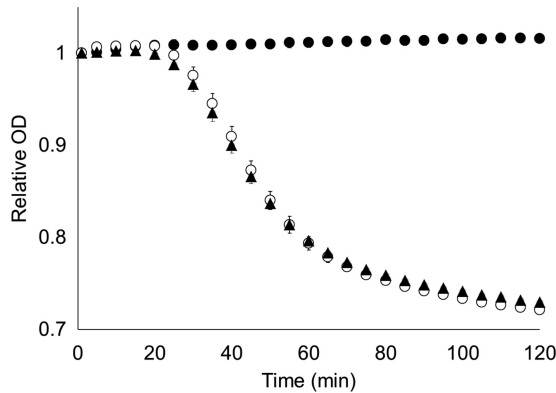


FIG 1 *P. larvae* spores germinate in artificial worker jelly. *P. larvae* strain ATCC 49843 spores were suspended in defined medium (●), artificial worker jelly (○), or uric acid plus L-tyrosine (▲). Data are shown for every 5 min for clarity. Spore germination was followed by decreases of the relative OD over time. Each error bar represents a standard deviation obtained from at least six independent measurements.

lus cereus germinate within 2 h in rich medium (39, 40). Similarly, *P. larvae* spores failed to germinate in a defined medium containing metabolites commonly used as germinants by *Bacillus* and *Clostridia* species (Fig. 1).

Honey bee larvae are fed royal or worker jelly that can be contaminated with *P. larvae* spores (41). Here, *P. larvae* spores were resuspended in a chemically defined medium (AWJ) that differs from worker jelly only in its pH value. The optical density of *P. larvae* spores suspended in AWJ decreased, indicating that the spores were germinating (Fig. 1). Spore germination was confirmed by Schaeffer-Fulton staining (30).

To determine the compounds that are necessary to trigger *P. larvae* spore germination, groups of compounds were systematically omitted from the AWJ medium. *P. larvae* spores germinated well in the absence of sugars and vitamins, suggesting that germination onset required uric acid and proteinogenic amino acid(s). Testing of individual amino acids showed that only L-tyrosine was able to synergize with uric acid to produce a sufficiently strong

germination response in *P. larvae* spores (Fig. 1). A mixture of uric acid and the remaining 19 proteinogenic amino acids induced negligible germination responses (data not shown).

To determine the effects of temperature on *P. larvae* spore germination, a range of 25 to 42°C was tested (Fig. 2A). At temperatures of <30°C, the germination rate of *P. larvae* spores was low. The maximal germination rates occurred at temperatures of >35°C. The ability of *P. larvae* spores to germinate was also tested at different pH values (Fig. 2B). Under acidic or basic conditions, spores failed to germinate. Germination was optimal near neutral pH.

Titration of L-tyrosine at a saturating uric acid concentration yielded an EC₅₀ of 1.2 mM for L-tyrosine activation of *P. larvae* spore germination (Fig. 3A). Titration of uric acid at a saturating L-tyrosine concentration yielded an EC₅₀ of 0.2 mM for uric acid activation of *P. larvae* spore germination (Fig. 3B). Both dose-response assays resulted in sigmoidal curves that passed the Durbin-Watson statistical test for autocorrelation.

Uric acid is a degradation product of purine catabolism. Hence, we tested the ability of purine analogs to act as cogerminants of *P. larvae* spores. Since L-tyrosine is the only amino acid able to trigger *P. larvae* spore germination, we also tested its stereoisomer (D-tyrosine) and its side chain (phenol) as cogerminants with uric acid. None of the compounds tested as a cogerminant was able to activate *P. larvae* spore germination (data not shown).

L-Tyrosine and purine analogs were also tested for their ability to inhibit *P. larvae* germination. None of the purine analogs tested inhibited uric acid–L-tyrosine-induced germination of *P. larvae* spores (data not shown). Similarly, D-tyrosine did not inhibit *P. larvae* spore germination. Of the compounds tested, only indole (the side chain of tryptophan) and phenol (the side chain of tyrosine) were able to inhibit *P. larvae* spore germination. Titrations of indole and phenol yielded IC₅₀s of 0.37 mM (Fig. 4A) and 0.46 mM (Fig. 4B), respectively, for the inhibition of *P. larvae* spore germination. 3-Methylindole, 5-methylindole, and 7-methylindole retained inhibitory properties, with IC₅₀s of 0.38, 0.37, and 0.28 mM, respectively (Table 1). In contrast, 1-N-methylindole

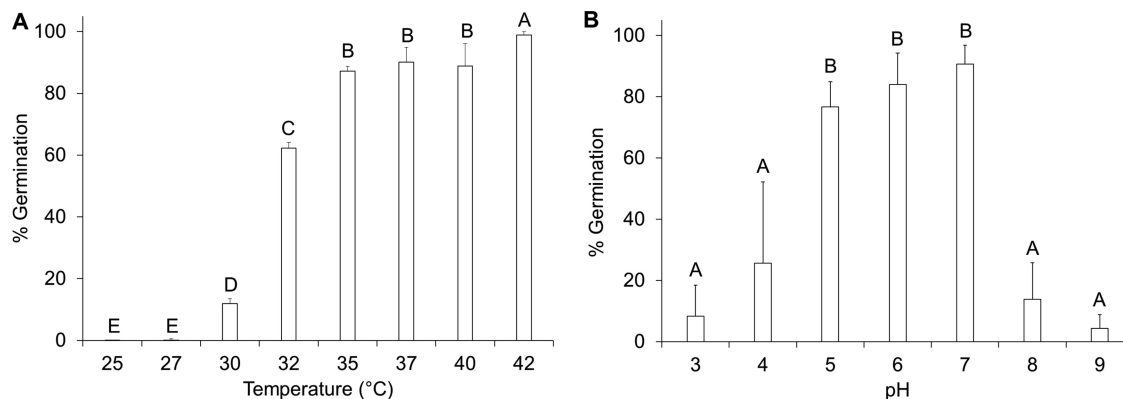


FIG 2 Effects of temperature and pH on *P. larvae* spore germination. (A) *P. larvae* spores were suspended in 3 mM L-tyrosine–3 mM uric acid and exposed individually to temperatures between 25 and 42°C. The maximum germination rate was set to 100% for spores germinated at 42°C. Percent germination for other conditions was calculated relative to 42°C. Each error bar represents a standard deviation obtained from at least six independent measurements. Columns that are labeled with different letters are statistically different ($P > 0.05$). (B) *P. larvae* spores were suspended in 3 mM L-tyrosine–3 mM uric acid and were exposed individually to pH values between 3 and 9. The maximum germination rate was set to 100% for spores germinated at pH 7. Percent germination for other conditions was calculated relative to pH 7. Each error bar represents a standard deviation obtained from at least six independent measurements. Columns that are labeled with different letters are statistically different ($P > 0.05$).

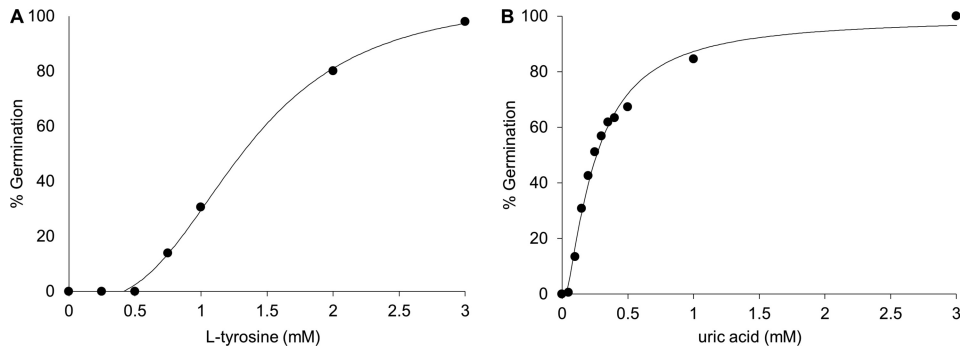


FIG 3 Calculation of 50% effective concentration for L-tyrosine and uric acid. (A) Dose-response curve of *P. larvae* spores germinated at a saturating concentration of uric acid and various concentrations of L-tyrosine. The EC₅₀ for L-tyrosine was determined based on these data. (B) Dose-response curve of *P. larvae* spores germinated at a saturating concentration of L-tyrosine and various concentrations of uric acid. The EC₅₀ for uric acid was determined based on these data.

did not activate or inhibit *P. larvae* spore germination. All dose-response assays resulted in sigmoidal curves that passed the Durbin-Watson statistical test for autocorrelation.

To test the generality of *P. larvae* spore response, we prepared spores from *P. larvae* subsp. *pulvificiens* strain ATCC 49843 and from an environmental AFB sample. Based on phenotypic characteristics, this environmental sample was identified as *P. larvae* subsp. *larvae* (13, 14). The spores of both *P. larvae* subspecies responded identically to L-tyrosine and uric acid. Their germination was similarly inhibited by indole and phenol (data not shown).

DISCUSSION

Bacterial spore germination is a critical step for infection onset in numerous hosts (42, 43). The nature of the spore germination signals has been studied widely in *Bacilli* and *Clostridia* (21, 27, 28, 44–50), but the triggers for *P. larvae* spore germination have not been identified. In order to cultivate *P. larvae*, specialized media have been produced (1, 31, 51–53). Under the best conditions, <10% of *P. larvae* spores plated on complex laboratory media germinate (54). In this study, we show that *P. larvae* spores specifically recognize L-tyrosine and uric acid as germinants.

P. larvae spores germinate at a sluggish pace at room temperature and thus can remain dormant on hive surfaces and beekeeping equipment for long periods of time. Honey bees are endothermic and use heat-producing muscle contractions to maintain

colony temperature at a narrow range, around 35°C (41). To develop correctly in the laboratory, reared honey bee larvae are maintained at a constant temperature of 35 to 37°C (55), which is also optimal for *P. larvae* spore germination.

Maximal germination of *P. larvae* spores was observed between pH 5 and 7, which matches the intestinal pH levels of both adult bees (pH 5.6 to 6.3) and bee larvae (pH 6.8) (56). Honey, nectar, pollen, and royal jelly are much more acidic (pH 3 to 4). We suggest that the acidity of these products may prevent *P. larvae* spores from germinating prematurely outside the honey bee gut.

Although the concentration of L-tyrosine in the bee larva gut is not known, honey bee larvae are fed either worker or royal jelly containing free L-tyrosine at a concentration of approximately 0.11 mM (57). Royal jelly also contains proteins that, upon digestion, can increase the concentration of L-tyrosine to 22 mM (57).

Once metabolized, proteinaceous materials are converted to uric acid, which is excreted as a waste product (58). Uric acid in the midgut of honey bee larvae must be at saturating concentrations, because when larvae first defecate, uric acid precipitates as large crystals (58). In fact, the appearance of uric acid crystals has been used as a marker for pupation onset (41) and insect infestation of stored grains (59).

The *Bacilli* and *Clostridia* spore germination responses result from complex interacting pathways (26, 60). Indeed, six different strategies have been described that integrate the multiple signals

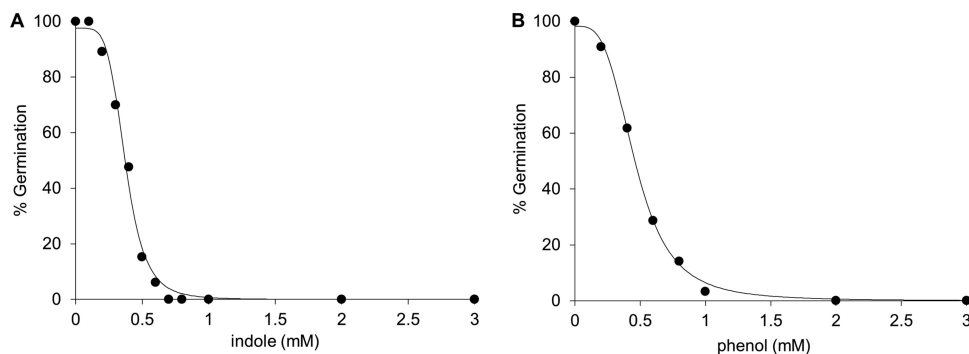
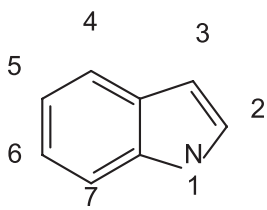


FIG 4 Calculation of 50% inhibitory concentration. (A) Dose-response curve of *P. larvae* spores germinated with L-tyrosine–uric acid in the presence of various concentrations of indole. The IC₅₀ for indole was determined based on these data. (B) Dose-response curve of *P. larvae* spores germinated with L-tyrosine–uric acid in the presence of various concentrations of phenol. The IC₅₀ for phenol was determined based on these data.

TABLE 1 Effects of indole methylation on *P. larvae* spore germination^a


Compound	IC ₅₀ (mM) ^b
Indole	0.37 (0.02)
Phenol	0.46 (0.02)
1-N-Methylindole	NA
3-Methylindole	0.38 (0.01)
5-Methylindole	0.37 (0.02)
7-Methylindole	0.28 (0.01)

^a *P. larvae* spores were incubated with various concentrations of indole analogs for 15 min prior to addition of 3 mM L-tyrosine–3 mM uric acid.

^b IC₅₀ was calculated by plotting percent germination versus indole analog concentration. Standard deviations are shown in parentheses. NA, no activity under the conditions tested.

required for spore germination (22). We showed recently that active germination pathways in *Bacillus* can cooperate or interfere with each other (61). In contrast, the simplicity of signals required for *P. larvae* spore germination can be described as a single integrator logical gate (22). By narrowing the germination signals to two germinants, *P. larvae* spores further ensure that germination occurs only in the larval gut, where amino acids and uric acid are abundant.

The midgut and the hindgut of honey bee larvae are disconnected until the final larval molt. Hence, in the AFB-susceptible young larvae, food and waste products will colocalize in the midgut (41). This will ensure that *P. larvae* spores are exposed simultaneously to high concentrations of uric acid and L-tyrosine, allowing for germination and infection onset. In contrast, in adult honey bees, amino acids are thought to be absorbed in the midgut, and uric acid is present only in the hindgut (58, 62). The spatial and temporal separation between food and waste products in adult honey bees will preclude *P. larvae* spores from detecting both germination signals, thus preventing germination (41).

Spore-formers can have strain-specific differences in their germination responses. For example, while adenosine is a germinant for *B. cereus* strain 3711, it inhibits inosine-mediated germination in *B. cereus* strain 569 (63, 64). *P. larvae* spores from the two subspecies show identical germination responses. Our results are consistent with polyphasic taxonomic studies that have found few differences between germination responses in *P. larvae* strains (14).

The spore germination response has been studied in other *Paenibacillus* species. Spores of *Paenibacillus polymyxa* germinate in response to the presence of fructose and alanine (48). The difference in germination responses between *P. larvae* and *P. polymyxa* was expected since *P. polymyxa* spores are associated with plant roots, where the concentration of uric acid is low. Other members of the *Paenibacillus* genus are insect pathogens that specifically target the larval stage. Indeed, *Paenibacillus popilliae* spores are sold commercially for the control of Japanese beetle infestation (65). It is tempting to speculate that uric acid can serve

as a general germination signal for insect larva pathogens. Indeed, while there are no other reports of bacterial spores that germinate in response to the presence of uric acid and L-tyrosine, *Bacillus fastidiosus* spore germination occurs in a medium containing only uric acid (66), while *Clostridium cylindrosporium* spores germinate in a medium containing bicarbonate, uric acid, and calcium (49). Both of these bacteria are present in poultry litter and bird droppings, where uric acid is abundant (49, 67). The ability to recognize uric acid and an amino acid cogerminant might allow bacteria to select for specific hosts.

Although D-amino acids are strong spore germination inhibitors of *Bacillus* species (68–72), D-tyrosine failed to inhibit *P. larvae* spore germination. Of the analogs tested, only indole (the side chain of tryptophan) and phenol (the side chain of tyrosine) inhibited *P. larvae* spore germination.

Indole and phenol might inhibit *P. larvae* spore germination through nonspecific binding to hydrophobic regions of the tyrosine or uric acid binding sites. Methylindole derivatives are more hydrophobic than indole derivatives, but none of these analogs show increased antigermination activity. On the contrary, methylation at the N-1 position eliminates antigermination activity, suggesting that the NH group of indole forms an essential hydrogen bond with the tyrosine and/or uric acid binding sites.

Indole is the last intermediate in the biosynthesis of tryptophan in plants and bacteria (73). Indole also acts as an intracellular and extracellular signal for virulence, biofilm formation, acid resistance, drug resistance, and sporulation in bacteria (74–79). Phenol, on the other hand, is toxic in its free form but is found as a functional group in many secondary metabolites of plants (80). Since honey bees gather pollen and nectar, which are very rich in phenolic compounds, it is possible that collected polyphenols and indole compounds can protect honey bee larvae from infection.

In conclusion, we have found the activators and inhibitors of *P. larvae* spore germination. We also present evidence to suggest that *P. larvae* spores have evolved to germinate only in the gut of the larvae and to remain dormant in food, exposed environments, and the adult bee.

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