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Melanin Pigment Formation and Increased UV Resistance in *Bacillus thuringiensis* Following High Temperature induction

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Summary

The pigment melanin is well known to protect against the damaging effects of UV radiation. In this study, we show that thirty-five of thirty-seven tested *Bacillus thuringiensis* strains possess the potential to produce melanin in the presence of L-tyrosin at elevated temperature (42 °C). These findings offer a method of protecting insecticidal toxins produced by *B. thuringiensis* from UV degredation and may therefore have important applications in the field of crop protection. Toxicity assays on *Heliothis armigera* suggested that the insecticidal activity of *B. thuringiensis* that produced melanin was significantly higher after UV irradiation than when melanin was not produced.

Key words: *Bacillus thuringiensis* – melanin – temperature inducing – UV irradiation

Introduction

The most widely used microbial pesticides are those based on preparations of the bacterium *Bacillus thuringiensis* [12, 15]. *B. thuringiensis* is a Gram-positive, spore-forming bacterium that produces highly specific insecticidal proteins, the δ-endotoxins. During sporulation, δ-endotoxin accumulates in an inactive protoxin form as a crystalline inclusion within the bacterial cells. If ingested by a susceptible insect, the crystals of protoxin dissolved and the δ-endotoxin is specifically cleaved by proteases in the insect gut. The resulting activated toxins recognize a specific receptor on the surface of larval midgut epithelial cells causing cell lysis and ultimately death of the insect [8]. In contrast to chemical insecticides, this microbial pesticide is safe to non-target organisms, poses no threat of pollution to the environment, and does not promote the development of a resistant insect population [15]. However, *B. thuringiensis* products are typically unstable in the environment after spraying because they are rapidly inactivated by exposure to sunlight [16]. Consequently, the duration of pest control is often too short and its use on many crops is not cost-effective because too many applications are required in order to successfully control insect population [1]. Therefore, the economic viability and acceptability of *B. thuringiensis* biopesticides depends on the potency of activity of the insecticidal toxins of the crystals.

Melanin is a dark brown-black pigment formed by the action of tyrosinase, a monoxygenase which catalyzes the conversion of L-tyrosine, via L-DOPA to dopachrome, which is subsequently polymerized to melanin via a series of nonenzymatic reactions [11]. In the last few years, there has been a growing interest in melanin research as some important functions have been recognized. Many reports focus on the ability of melanin to protect against radiation damage. Melanin absorbs light at all wavelengths and reaches its maximum absorbance in the UV range. In particular, melanin's ability to increase UV resistance and preserve the insecticidal activity of *B. thuringiensis* products has been reported [9, 13, 14, 18].

We first report here that the majority of the *B. thuringiensis* strains possess the potential to produce melanin by high temperature (42 °C) induction. Our research demonstrates that melanin increases the UV resistance of *B. thuringiensis*, providing a new approach for more effective use of *B. thuringiensis* preparations in crop protection.

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Materials and Methods

Bacterial strains

All the *B. thuringiensis* strains except 94001 in Table 1 were obtained from the China Center For Type Culture Collection. *B. thuringiensis* subsp. *kustaki* 94001, which was kindly provided by Hubei B.t. Research and Development Center, was used for bioassay.

Media

NCM media contains per liter: 5 g bactotryptone, 3 g bactoyeastextract, 0.7 mM $CaCl_2$, 0.05 mM $MnCl_2$, 1 mM $MgCl_2$, pH 7.2. The medium was sterilized by autoclaving for 30 min at 0.07 MPa in a liquid cycle. Upon cooling, 0.1% tyrosine was added to promote melanin production and 1.5% agar for solid medium.

Synthesis and quantification of L-DOPA

Vitamin C and L-tyrosine were added to cultures of *B. thuringiensis* grown in LB medium for 14 hr at pH 5.5 at a rate of three times every two hours until the final concentration of vitamin C and L-tyrosine reached 5.5 mg ml^{-1} and 5 mg ml^{-1}, respectively. The role of vitamin C is to inhibit the polymerization of L-dopa to melanin via a series of non-enzymatic reactions. The bacteria were cultured continuously for another 12 hr incubation, the cell mass was pelleted by centrifugation at 3792 g for 10 min and the supernatant analyzed for L-DOPA [10].

Preparation of *B. thuringiensis* samples

The *B. thuringiensis* was cultured in NCM medium containing L-tyrosine at 42 °C to produce melanin and then transfered to 30 °C till the crystalline toxin was released from the cells. Parallel cultures of the same strain grown in NCM medium without L-tyrosine with the same condition did not produce the melanin.

UV irradiation of *B. thuringiensis* samples

B. thuringiensis samples were irradiated in a UV luminaire box, which delivered a wavelength of 245 nm. Spore-crystal preparations (2 ml) of *B. thuringiensis* samples were sprayed onto petri dishes (radius = 3 cm) opened under the UV luminaire at a distance of 20 cm from the source, and irradiated for 1 hr, 2 hr and 3 hr. The energy output of UV source is 14.83 J/cm^2, 29.65 J/cm^2 and 44.48 J/cm^2, respectively, Which was measured with a uvx digital radiometer (UVP, Inc). The irradiated spores-crystals were analyzed by using SDS-PAGE and assayed using a *Heliothis armigera* bioassay as described below.

Preparation and electrophoresis of crystal protein

The irradiated spore-crystal suspension was lysed with 0.1 N NaOH for 3 min, boiled at 100 °C in loading buffer for 5 min, and loaded onto an 8% acrylamide gel.

Insecticidal activity bioassays

Insect bioassays were performed with first instar larvae of *Heliothis armigera*. The *Heliothis armigera* larvae were fed with an artificial diet [20] containing 5 to 6 successive dilutions of the *B. thuringiensis* samples to be tested. A total of 48 larvae were fed with each dilution. The larvae were placed in individual wells and incubated at 30 °C. Mortality was scored after 72 hours. The concentration of spore-crystal gave the 50% mortality (LC_{50}) determined by log-probit analysis [4] The potency of each sample against *Heliothis armigera* larvae was expressed as international units (IU) per milligram of *B. thuringiensis* samples and calculated with the following formula: LC_{50} of the standard (CS3ab-1991, Hubei B.t. Research and Development Center)/ LC_{50} of the sample]*potency of the standard (15000 IU/mg)[5].

Table 1. Capacity and minimum time of *B. thuringiensis* strains producing melanin following induction at 42 °C.

Strains	Characteristics	Melanin producing capacity	Minimum time of producing melanin (hour)
4Q7	*B. thuringiensis* subsp. *israelensis*, Cry^-	+++++	16
171	*B. thuringiensis* subsp. *kustaki*, Cry^-	++	30
AB91003	*B. thuringiensis* subsp. *finitimus*	+	48
AB91004	*B. thuringiensis* subsp. *alesti*	+++++	16
AB91006	*B. thuringiensis* subsp. *dendrolimus*	+++	22
AB91007	*B. thuringiensis* subsp. *sotto*	+++++	35
AB91010	*B. thuringiensis* subsp. *canadensis*	+++++	16
AB91011	*B. thuringiensis* subsp. *entomocidus*	++++	30
AB91012	*B. thuringiensis* subsp. *subtoxicus*	+	48
AB91014	*B. thuringiensis* subsp. *tolworthi*	+++	40
AB91015	*B. thuringiensis* subsp. *darmstadiensis*	+	48
AB92029	*B. thuringiensis* subsp. *toumanoffi*	+	48
AB 92030	*B. thuringiensis* subsp. *kyushuensis*	+	48
AB92031	*B. thuringiensis* subsp. *dakota*	++++	18
AB92032	*B. thuringiensis* subsp. *indianae*	++++	20
AB92033	*B. thuringiensis* subsp. *tokohuensis*	+++	22
AB92034	*B. thuringiensis* subsp. *humamotoensis*	++++	16
AB92035	*B. thuringiensis* subsp. *tochigiensis*	+	48
AB92036	*B. thuringiensis* subsp. *pondicheriensis*	+++	24
AB92038	*B. thuringiensis* subsp. *shandongiensis*	+++	40
AB92039	*B. thuringiensis* subsp. *noeleonensis*	+	36
AB92040	*B. thuringiensis* subsp. *koreanensis*	+	48
AB92041	*B. thuringiensis* subsp. *mexicanensis*	+++++	16
AB92042	*B. thuringiensis* subsp. *siloensis*	+++	16
AB92043	*B. thuringiensis* subsp. *morrisoni*	+++	22
AB93062	*B. thuringiensis* snbsp. *galleriae*	+++++	16
AB91023	*B. thuringiensis* snbsp. *galleriae*	+++++	16
AB91024	*B. thuringiensis* subsp. *galleriae*	++	24
AB91005	*B. thuringiensis* subsp. *kurstaki*	+	48
AB90010	*B. thuringiensis* subsp. *kurstaki*	++++	36
94001	*B. thuringiensis* subsp. *kurstaki*	++++	24
AB91008	*B. thuringiensis* subsp. *kenyae*	+++	48
AB91019	*B. thuringiensis* subsp. *israelensis*	+++	36
AB92027	*B. thuringiensis* subsp. *nigerinsise*	–	48
AB91018	*B. thuringiensis* subsp. *parkistani*	++	16
AB94047	*B. thuringiensis* subspecies did not identified	+++	20
AB92079	*B. thuringiensis* subspecies did not identified	+++	20
AB91022	*B. thuringiensis* subspecies did not identified	–	48

Note: Number of plus sign (+) represents the capacity of *B. thuringiensis* strains to produce melanin. *B. thuringiensis* strains cannot produce melanin is noted with subtraction sign (–).

Results

Melanin formation

Thirty-five of the thirty-seven *B. thuringiensis* strains tested were all found to produce a dark-brown, diffusible pigment following incubation at 42 °C for several hours. In contrast, the same strains fail to produce melanin when grown at 30 °C (Fig. 1). The formation of this pigment depended on the presence of L-tyrosine in the culture medium. The capacity of *B. thuringiensis* strains to produce melanin is shown in Table 1. The optimal pH for melanin formation of *B. thuringiensis* is pH 7.0. The black-brown pigment from *B. thuringiensis* was confirmed to be melanin through characterization by infrared (IR) and ultraviolet spectroscopic analyses (data not shown). The characteristic absorbance peak of the black-brown pigment produced by *B. thuringiensis* was deduced to be melanin when compared to a commercially available source sample (Sigma).

Production of L-DOPA by *B. thuringiensis*

Formation of melanin pigment by *B. thuringiensis* is a new phenomenon. First, the biosynthetic pathway for melanin must be determined. The formation of this pigment depended on the presence of L-tyrosine in the culture medium. Therefore, we can deduce that melanin is formed by the action of tyrosinase [17]. Furthermore, the presence of L-DOPA in the supernatant of *B. thuringiensis* cultured at 42 °C was observed (see Materials and Methods), confirming our hypothesis that the black pigment produced by *B. thuringiensis* is synthesised via L-DOPA. The maxium measured concentration of L-DOPA in our study is 43.75 $\mu g \cdot ml^{-1}$.

Effect of melanin on protecting crystals from degradation after UV irradiation

B. thuringiensis produces crystalline protein inclusions which are a key component of toxicity during sporulation. However, the crystals are easily degraded by exposure to UV irradiation. Protecting crystals from degradation is thus a major factor in improving the efficacy and economics viability of the bioinsecticide. In this research we have detected the ability of melanin to protect the crystalline toxin from inactivation by UV irradiation. The *B. thuringiensis* spore-crystal suspensions, in the presence and absence of melanin, were irradiated by UV for different times and then electrophoresed (see Materials and Methods). The samples in which melanin was produced were found be less damaged as compared to the control sample (Fig. 2).

Insecticidal activity of *B. thuringiensis* strains 94001 with or without melanin after UV irradiation

The insecticidal activity of *B. thuringiensis* strains 94001with and without melanin were assayed on *Heliothis armigera* after irradiation. From the data in Table 2, we observed that the LC_{50} was significantly higher for *B. thuringiensis* strain 94001 without melanin than for the same strain with melanin. Accordingly, the potency of

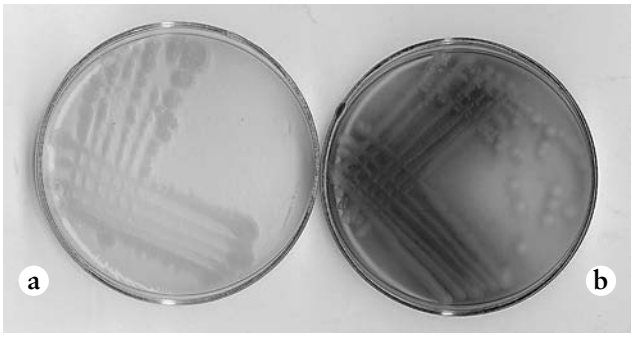

Fig. 1. Pattern of melanin production for (**a**) *B. thuringiensis* cultured on NCM plate containing 0.1% tyrosine at 30 °C. (**b**) *B. thuringiensis* cultured on NCM plate containing 0.1% tyrosine at 42 °C.

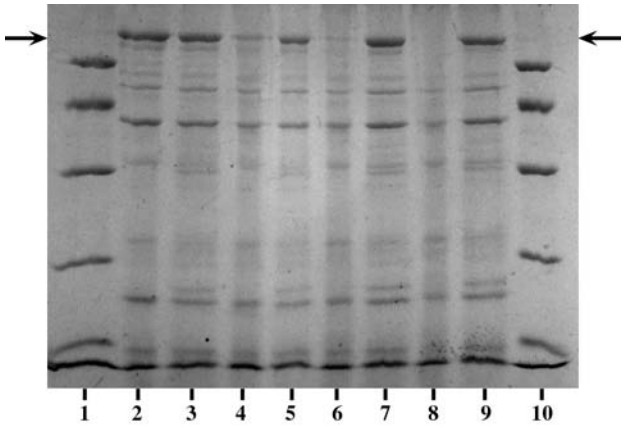

Fig. 2. SDS-PAGE analysis of spore-crystal preparations from *B. thuringiensis* subsp. *kustaki* strain with and without melanin. Lanes 1, 10: protein marks (from top to bottom: 97, 66, 43, 31 and 20 kDa); Lanes 2, 4, 6, 8: *B. thuringiensis* spore-crystal suspensions without melanin were irradiated by UV for 0, 1, 2 and 3 hours, respectively. Lanes 3, 5, 7, 9: *B. thuringiensis* spore-crystal suspensions with melanin were irradiated by UV for 0, 1, 2 and 3 hours, respectively. Arrows indicate the 130–140 kDa crystal components.

Table 2. Insecticidal activity of *B. thuringiensis* strains 94001against *Heliothis armigera* larvae after UV irradiation.

Samples	LC_{50} µg/mlPotencyIU/mg							
	0 hr	1 hr	2 hr	3 hr	0 hr	1 hr	2 hr	3 hr
B. thuringiensis with melanin	29	30	60	66	53276	51500	23409	22391
B. thuringiensis without melanin	28	52	74	81	55179	29711	21164	19074

B. thuringiensis strains 94001without melanin were lower after irradiation as compared to the same sample with melanin. Therefore, we conclude that the melanin produced by *B. thuringiensis* can improve the duration of *B. thuringiensis* preparations after UV radiation.

Discussion

Studies have shown the wide presence of melanin in fungi, plants, insects and mammalian cells. The role of melanin is thought to improve the host's survival and competence [3].

In this paper we have described a new phenomenon in which the majority of wide-type *B. thuringiensis* strains can produce a dark-brown pigment after being cultured in the presence of tyrosine at 42 °C for several hours. On the basis of all our results, we conclude that *B. thuringiensis* does have the potential to produce melanin although it fails to do so in the normal state.

The sunlight-mediated degradation of *B. thuringiensis* preparations drastically lowers the toxicity of these biopesticides in the field. Various techniques have been reported to protect *B. thuringiensis* preparations from solar inactivation by encapsulation [19] and addition of a variety of UV protectants [6, 7], these approaches all have negative ecological impact [2]. In this research we demonstrate an attractive alternative which utilizes the UV-protective properties of melanin, which can be produced by *B. thuringiensis* in an efficient and safe manner. This unique method of generating a UV protectant avoids the addition of exogenous material to *B. thuringiensis* preparations, making, it an ecologically safer and more cost effective.

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References

1. Cannon, R. J. C.: *Bacillus thuringiensis* in pest control, p.190-200. In: Biological control: benefits and risks (Hokkanen, H. M. T., and Lynch, T. M., eds.). Cambridge University Press, Cambridge, England (1995).
2. Cohen, E., Rozen, H., Joseph, T., Braun S., Margulies, L.: Photoprotection of *Bacillus thuringiensis* kurstaki from ultraviolet irradiation. J. Invertebr. Pathol. 57, 343–351.
3. Coyne, V. E., and Al-Harthi, L.: Induction of melanin biosysthesis in *Vibrio cholerae*. Appl. Environ. Microbiol. 58, 2861–2865 (1992).
4. Daum, R. J.: A revision of two computer programs for probit analysis. Bull. Entomol. Soc. Am. 16, 10–15 (1970).
5. Dulmage, H. T.: Insecticidal activity of isolate of *Bacillus thuringiensis* and their potential for pest control. In: Microbial Control of Pest and Plant Diseases 1970–1980 (Burges, H. D., ed.), pp. 193–222. Academic Press, New York (1981).
6. Dunkle, R. L., and Shasha, B. S.: Starch encapsulated *Bacillus thuringiensis*: A potential new method for increasing environmental stability of entomopathogens. Environ. Entomol. 17, 120–126 (1988).
7. Dunkle, R. L., and Shasha, B. S.: Response of starch encapsulated *Bacillus thuringiensis* containing UV screens to sunlinght. Environ. Entomol. 18, 1035–1041 (1989).
8. Entwistle, P. F.,Corey, J. S., Bayley, M. T., and Higgs, S. (ed.): *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. John Wiley and Sons Ltd., Chichester, England (1993).
9. Hoti, S. L., Balaraman, K.: Formation of melanin pigment by a mutant of *Bacillus thuringiensis* H-14. J. Gen. Microbiol. 139, 2365–2369 (1993).
10. Hu, X., Peng, Z., Shen, P.: Selection and mutagenesis of mutants with high tyrosinase activity. J. Amino. Acid 13, 9–13 (1991).
11. Ito, S., Wakamatsu, K., and Ozeki, H.: Chemical analysis of melanins and its application to the study of the regulation of melanogenesis. Pigment Cell Res. 13, 103–109 (2000).
12. Lambert, B., and Peferoen, M.: Insecticidal promise of *Bacillus thuringiensis*. Facts and mysteries about a successful biopesticide. Bioscience 42, 112–122 (1992).
13. Liu, Yu-Tien, Sui, Meng-Jiun, Ji, Dar-Der, Wu, I-Huan, Chou, Chin-Chi, and Chu, cheng-chen: Protection from ultraviolet irradiation by melanin of mosquitocidal activity of *Bacillus thuringiensis var. Israeliensis*. J. Invert. Pathol. 62, 131–136 (1993).
14. Patel, K. R.,Wyman, J. A., Patel, K. A., andBurden, B. J.: A mutant of *Bacillus thuringiensis* Producing a Dark-Brown Pigment with lncreased UV resistance and lnsecticidal Activity. J. lnverteb. Pathol. 67, 120–124 (1996).
15. Powell, K. A.: The commercial exploitation of microorganism in agriculture, p. 441–459. In: Exploitation of microorganisms (D. G. Jones. ed.). Chapman and Hall, London, England (1993).
16. Pusztai, M., Fast, M., Gringorten, L., Kaplan, H., Lessard, T., and Carey, P.: The mechanism of sunlight-mediated inactivation of *Bacillus thuringiensis* crystals. Biochem. J. 273, 43–47 (1991).
17. Riley, P. A.: Mechanistic aspects of the control of tyrosinase activity. Pigment Cell Res. 6, 182–185 (1993).
18. Saxena, D., Ben-Dov, E., Manasherob, R., Barak, Z., Boussiba, A., Zaritsky, A.: A UV tolerant mutant of *Bacillus thuringiensis* subsp. *kurstaki* producing melanin. Curr. Microbiol. 44, 25–30 (2002).
19. Tamez-Guerra, P., McGuire, M. R.,. Behle, R. W., Shasha, B. S., Wong, L. J.: Assessment of microencapsulated formulations for improved residual activity of *Bacillus thuringiensis*. J. Econ. Entomol. 93, 219–225 (2000).
20. Zhong Liansheng, Xie Tianjian, Wu Jixing: Study on Bioassay for toxicity of *B. thuringiensis* preparations using *Heliothis armigera* as tested insect. Chin. J. Biol. control 6, 1–5 (1990).

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