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ISOLATION AND PARTIAL CHARACTERIZATION OF A MUTANT OF *BACILLUS THURINGIENSIS* PRODUCING MELANIN

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SHORT COMMUNICATION

ABSTRACT

A mutant (407-P) of *Bacillus thuringiensis* subsp. *thuringiensis* strain 407 producing a melanin was obtained after treatment with the mutagenic agent ethyl-methane-sulfonate. Several microbiological and biochemical properties of the two strains were analyzed and the results were similar. The mutant 407-P was also incorporated into non-sterilized soil samples, recovered, easily identified, and quantified, what enables its use in ecology of *B. thuringiensis*.

Key-words: *Bacillus thuringiensis*, melanin, mutation, ecology

Bacillus thuringiensis is a spore-forming bacterium that produces highly specific insecticidal proteins, termed Cry proteins (7). Numerous Cry protein genes (called *cry* genes) have been classified into groups (http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/). During sporulation, Cry proteins accumulate as crystalline inclusions within the cell. These crystals, when ingested by susceptible insects, are dissolved releasing monomers of the Cry proteins, followed by the proteolitic processing of the protoxin by midgut proteases, releasing the Cry toxin in its active form. The actived toxin binds to specific receptors on the apical brush border of the midgut microvillae of susceptible insects causing lysis of midgut epithelial cells and death of insect larvae (20).

Because of this mode of action with high specificity, microbial pesticides derived from *B. thuringiensis* present many advantages such as safety for non-target organisms, high specificity, low development of pest resistance and low environmental pollution. Therefore, *B. thuringiensis*-derived microbial pesticides are the most widely used in the world (4). However, the use of *B. thuringiensis* products is limited because the spores and toxins are highly sensitive to Ultra-violet (UV) radiation (8,9,15) and because different formulations are inadequately stable under field conditions and rapidly lose their biological activities (2,23).

Several investigators have reported that the production of melanin by various microorganisms protects their susceptibility to damage caused by UV and ionizing radiation (1,16,17). Melanins are natural screens against both UV and visible light irradiation. They also provide additional protection against desiccation and attack by cell wall lytic enzymes (18).

Three methods have been utilized for obtaining *B. thuringiensis* products protected by pigments. I) Liu *et al.* (13) enhanced the UV resistance of *B. thuringiensis* by mixing *B. thuringiensis* with melanin produced by the fermentation of *Streptomyces lividans* 66 harboring a recombinant plasmid bearing the respective *mel* gene; II) Ruan *et al.* (17) constructed a recombinant *B. thuringiensis* strain expressing a novel *mel* gene under the control of the *cry*3A gene expression system; III) Hoti and Balaraman (10) obtained a mutant of *B. thuringiensis* producing a dark-brown pigment, identified as melanin, by inducing mutagenesis.

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This study describes a method for obtaining a pigment-producing mutant of *B. thuringiensis* subsp. *thuringiensis* 407, utilizing ethylmethane-sulfonate (EMS). The nature of the pigment and the microbiological and biochemical characterization of the mutant supports its proposed application in the photoprotection of products derived from *B. thuringiensis* and in ecology studies as well.

B. thuringiensis subsp. *thuringiensis* 407 was mutagenized as described by Lecadet *et al.* (12) with some modifications. A suspension of spores (3×10^8 mL^{-1}) in sterile distilled water was thereby used, and EMS was then added to give a concentration of 10% v/v. The spores were incubated at 37ºC for 40 min. Afterward, the spores were washed twice with sterile water by centrifugation and ressuspended in 5 mL of sterile distilled water. Serial dilutions of the suspension were transferred onto Bacto Peptone BP (11) medium agar plates and incubated at 30ºC for three days. The colonies formed were then selected based on pigment production. A mutant of *B. thuringiensis* subsp. *thuringiensis* 407 producing a dark-brown diffusible pigment was isolated after morphological screening of the colonies and designated *B. thuringiensis* subsp. *thuringiensis* 407-P (Fig. 1).

The strain 407 and the mutant 407-P were grown in different complex media: nutrient broth, Luria Bertoni broth (LB) and BP. Pigment production was determined in these media having pH adjusted to 4; 5; 6; 7; 8, and 9, either in broth cultures, with shaking at 200 rpm, or in agar plates at 30ºC, for three days. The mutant 407-P produced a dark-brown diffusible pigment both in nutrient broth, BP, and LB in liquid cultures and on agar plates. In liquid BP and LB media, pigment production started after 24 h and continued up to 48 h. In nutrient broth, only weak production of pigment was detected after 72 h of culture, which began only after 48 h. Sporulation and crystal formation were monitored by phase-contrast microscopy, and were detected after 48 h in BP medium for both 407 and 407-P strains. For all media tested, the final pH of the cultures of the wild type strain was similar to the cultures of the 407-P mutant, and the optimum pH for pigment synthesis was 7. Similar results were shown in other studies (9,10) that tested for pigment production in several media and under various conditions. The results demonstrated that various factors affect pigment production, such as bacterial growth rate, medium composition and availability of a specific substrate.

For pigment purification and identification, cultures of 407-P strain were grown in LB medium at 30ºC and 200 rpm for 24 h. Pigment was extracted from the supernatant based on the method described by Espinasse *et al.* (8). Pigment was precipitated by adding 1M HCl to the supernatant, pelleted by centrifugation (10 min, 5000 × *g*), ethanol washed and dried. The extracted pigment was insoluble in hot and cold water, mineral acids, and solvents such as acetone and ethanol. It was completely solubilized by treatment with hot alkali (5M KOH) solution and upon the addition of 0.1M $FeCl_3$, a flocculent brown precipitate was formed. A similar pigmented phenotype was previously described for two different *B. thuringiensis* subspecies exposed to mutagenic agents (10,16,19).

The UV and visible absorption spectra of the pigment was obtained by spectrophotometer (Beckman DU640, Beckman, Coulter, Inc., Fullerton, CA, U.S.A.) scanning (Fig. 2) with the maximum absorbance at 205 nm. Our results are similar to that of the melanin produced by other *B. thuringiensis* strains. Although different mutagenic agents have been used to induce the production of melanin, such as UV light (16,19) and N-methyl-N'nitro-N-nitrosoguanidine (NTG) (10,16,19). This is the first study that describes the utilization of EMS as the mutagenic agent to obtain melanin-producing mutants in *B. thuringiensis*.

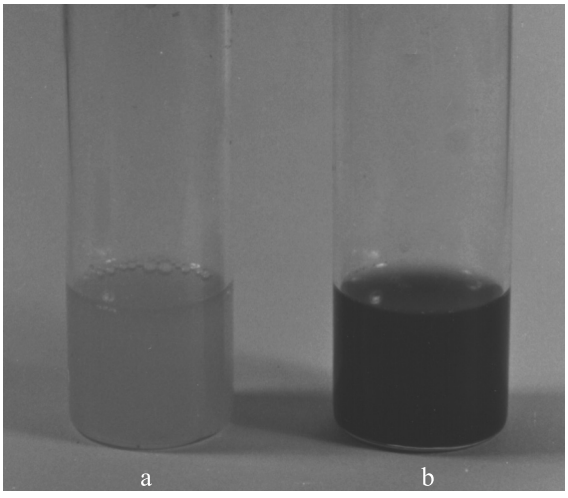

Figure 1. *Bacillus thuringiensis* subsp. *thuringiensis* 407 wild type strain (a) and the mutant 407-P producing pigment (b) in liquid LB medium.

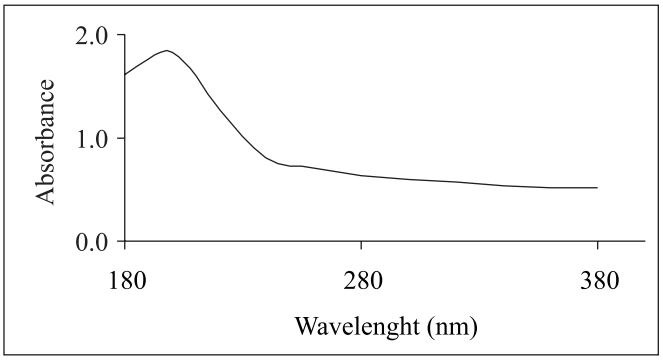

Figure 2. UV and visible spectra of the pigment from *B. thuringiensis* subsp. *thuringiensis* 407-P.

For monitoring the multiplication of 407-P and wild-type strains growth curve were performed. A volume of 3% of a pre-culture 0.6 at 600 nm was added to BP medium at 30ºC with shaking at 200 rpm and incubated for 15 h. Samples were taken each hour for optical density measurements and the setup of sporulation was monitored by phase-contrast microscopy. The results show that the growth curve of both 407-P and wild-type strains were identical with a reduced lag-phase and an increased log phase.

Susceptibility tests with commonly used antimicrobial agents were performed on LB agar by disk diffusion technique. The antibiotic disks (Cecon, São Paulo, Brazil) used were 10 µg ampicillin, 30 µg cefotaxime, 30 µg ceftriaxone, 2 µg clindamycin, 15 µg erythromycin, 30 µg kanamycin sulfate, 2 µg lincomycin, 1 µg oxacillin, IU penicillin G 10 and 30 µg vancomycin. *Escherichia coli* ATCC 25922 was used as control for bacterial growth and potency of antibiotics on disks. Inhibition disk zone sizes, interpreted according to the manufacturer's instructions, showed similar spectra for both mutant and the wild-type strains. Both were sensitive to clindamycin, erythromycin, kanamycin sulfate, vancomycin, and resistant to ampicillin, oxacillin, penicillin G, cefotaxime, ceftriaxone, and lincomycin.

Hemolytic activity was determined on sheep blood agar plates at 30ºC (24). The hemolytic halos generated were analyzed and compared in intensity and in shape over time after 15 h. The hemolytic activity of each strain (wild-type and mutant) was estimated twice and each replicate was classified as 'blind' with respect to the previous one. Gelatin-agar assays were performed for screening protease production in plates containing 1% (w/v) gelatin and 1.5% (w/v) agar. Spores were spotted on agar and, after three days of growth, the plate was flooded with 15% (w/v) $HgCl_2$ in 2M HCl. The unprecipitated clear zone showed the hydrolysis of gelatin due to the production of proteases. Protease production was measured as the ratio of the clear zone and of the colony diameters. These two tests were conducted to identify possible mutations in the pleiotropic activator PlcR, which regulates the production of extracellular proteins that are potentially involved in virulence, as phospholipases, proteases and hemolytic and non-hemolytic enterotoxins (21,25). Both tests were positive for both strains, including the detection of hemolythic activity and the production of proteases. This result indicates that there was no alteration in the expression of this important regulon, which regulates the expression of more than 100 genes.

Wild type and the mutant strains were tested for several biochemical reactions: phenylalanine deaminase, nitrate reduction, glucose fermentation with production of acid and gas, mannitol fermentation, and amylase production (3,14). Strains 407 and 407-P showed positive results for nitrate reduction, glucose fermentation with production of acid and gas and amylase production assays, and negative results for phenylalanine deaminase and mannitol fermentation assays. These results indicate that there was no significant alteration in metabolic pathways responsibles for acquisition and consumption of organic and inorganic nutrients available in the environment.

In this study a melanin-producing mutant of *B. thuringiensis* (407-P) was evaluated with respect to the ability of growing in different complex media. Change in culture pH, as well as sporulation and crystal production were monitored. In addition, susceptibility to several antimicrobial agents, the production of extracellular virulence factors (hemolysins and proteases) and the microbial properties of consumption and degradation of aminoacids and carbohydrates were also evaluated. No phenotypic alteration was detected in any of those traits. Therefore, it is suggested the utilization of the 407-P mutant to protect *B. thuringiensis* formulations from solar inactivation in the field, which appears to be one of the major environmental factors in the loss of their biological activity. Although various techniques have been reported to achieve the protection of *B. thuringiensis* formulations from solar inactivation (2,5,6,22), melanin offers the advantage that it is a natural product that is easily biodegraded, thereby avoiding soil and water pollution.

The second application of the 407-P mutant is the study of the ecology of *B. thuringiensis*. In order to confirm this application, tests were conducted to potentially detect this mutant in non-sterilized environmental samples. Cells of the 407-P strain at exponential growth were suspended in 0.85% NaCl and incorporated into 35 g of soil in Petri dishes, to obtain 10^8 cells g^{-1} of soil. The moisture content was kept at 80% of field capacity (33 g water per 100 g of dry soil) by adding sterile water, and the soil samples were incubated at 30ºC for one week. In all experiments, non-sterilized soil samples were used without pH correction and with no nutrients addition. Spores were extracted after one, two and three days, by mixing 3.2 g of soil with 18.8 mL of saline and shaking for 10 min in a pendular shaker. After heat treatment (70ºC, 20 min), appropriate dilutions were plated on BP agar and incubated at 30ºC for three days. The same treatment was made for soil samples without mutant cells addition. All the colonies obtained after heat treatment of the soil samples had *Bacillus*-like morphology. Pigment-producing and non pigment-producing colonies were observed, making it possible to identify and quantify the presence of the strain introduced into the soil samples. No pigment producing colonies were observed in the control experiments.

This result shows that pigment-producing *B. thuringiensis* strains can be introduced into environmental such as sample soils, insects, and water and easily monitored, without antibiotics marker utilization. The ecology of *B. thuringiensis* depends largely on studies under environmental conditions. Therefore, pigment-producing strains can be an important tool for studying the ecology of *B. thuringiensis*, with respect to growth and survival, persistence of spores in the environment, and the ability of spores to spread to surrounding areas.

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RESUMO

Isolamento e caracterização parcial de um mutante de *Bacillus thuringiensis* produtor de melanina

Um mutante (407-P) da linhagem *Bacillus thuringiensis* subsp. *thuringiensis* 407 produtor de melanina foi obtido após tratamento com o agente mutagênico etil-metano-sulfonato. Diversas propriedades microbiológicas e bioquímicas das duas linhagens foram analisadas e os resultados foram similares. O mutante 407-P foi incorporado em amostras de solo não esterilizado, recuperado, facilmente identificado e quantificado, possibilitando seu uso em estudos de ecologia de *B. thuringiensis*.

Palavras-chave: *Bacillus thuringiensis*, melanina, mutação, ecologia

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