Formation of melanin pigment by a mutant of *Bacillus thuringiensis* H-14

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A mutant of *Bacillus thuringiensis* H-14 produced a dark brown pigment during sporulation. Production of the pigment depended on the nutritional properties of the growth medium. The pigment was identified as melanin, based on chemical tests and its infra-red spectrum. Incorporation of L-tyrosine in the culture medium enhanced the level of melanin production, and L-3,4-dihydroxyphenylalanine (L-DOPA) was detected in the culture broth during the late-exponential phase of growth. This indicates that the pathway of melanin synthesis is from L-tyrosine, via L-DOPA, to melanin.

Introduction

Bacillus thuringiensis is an entomopathogenic, aerobic spore-forming bacterium (Buchanan & Gibbons, 1974). Strains of this species belonging to serotype H-14 have been recognized as potential candidates for use in the control of mosquitoes and blackflies (Lacey & Lacey, 1990). We have isolated a mutant of *B. thuringiensis* H-14 which produces a dark-brown pigment. The mutant is highly toxic to mosquito larvae and produces toxin much earlier than the parent strain (Balaraman & Hoti, 1987). The mutant has been used in field studies (Hoti & Balaraman, 1991). However, the nature of the dark-brown pigment was not studied.

Oxidative polymerization of phenolic compounds such as catechol and 1,8-dihydroxynaphthalene (allomelanins) (Allport & Bu'Lock, 1958), DOPA (eumelanins) and cysteinyl-DOPA (pheomelanins) (Sarna *et al.*, 1984) leads to the formation of brown-black pigments called melanins. The eumelanins and pheomelanins are found mainly in animal species, whereas the allomelanins are found in plants and micro-organisms (Nicolaus *et al.*, 1964). Bacteria belonging to the genera *Mycobacterium* (Prabhakaran *et al.*, 1968), *Bacillus* (Aronson & Wermus, 1965), *Vibrio* (Ivins & Holmes, 1980), *Rhizobium* (Beynon *et al.*, 1980), *Pseudomonas* (Delafield *et al.*, 1965; Ogunnariuo & Hamilton-Miller, 1975), *Micrococcus* (Mung & Kondrat'eva, 1981), *Gluconobacter* (Pavelenko *et al.*, 1981), *Azotobacter* (Sen & Sen, 1965) and

Azospirillum (Lakshmi & Neyra, 1987), have been reported to form melanins. In the present study we attempted to characterize the dark-brown pigment produced by the mutant of *B. thuringiensis* H-14.

Methods

Micro-organisms. A wild-type strain of *B. thuringiensis* H-14 (VCRC B17) (Balaraman *et al.*, 1981) and its mutant VCRC MB24, derived by chemical mutagenesis (Balaraman & Hoti, 1987), were used in this study. Both the strains were maintained on casein/glycerol (CL) medium (Yoshiharu *et al.*, 1982) at 4 °C.

Dynamics of melanin production. Cells were grown in potassium mineral salt (KMS) broth (Meenakshi & Jayaraman, 1979) on a rotary shaker at 250 r.p.m. at 30 ± 2 °C. Samples of cultures growing at 30 °C were taken at intervals and incubated at 4 °C for 5 d, to allow the formation of the colour. The cell mass was then removed from culture samples by centrifugation at 6000 r.p.m. for 15 min and the concentration of the pigment was measured in a spectrophotometer at 540 nm (Bull, 1970), using commercial melanin (Sigma) as a standard. Vegetative growth was monitored by nephelometry of cultures at 600 nm, and sporulation by light microscopy and by plating serially diluted samples on KMS agar plates, after heat treatment at 80 °C for 20 min (Petras & Casida, 1985).

Identification of the pigment. The cell mass was removed by centrifugation and the pigment in the supernatant was precipitated by lowering the pH to 3 using 1 M-HCl. The precipitate was pelleted by centrifugation at 10000 r.p.m. for 10 min and lyophilized (Ivins & Holmes, 1980).

The dry pigment was subjected to various chemical tests and infra-red (IR) spectroscopy, which are diagnostic of melanin (Bull, 1970; Ellis & Griffiths, 1974; Lakshmi & Neyra, 1987). For IR spectroscopy the pigment and potassium bromide were powdered together and the mixture was moulded into a pellet *in vacuo*. The IR spectrum of this pellet was recorded in a Perkin-Elmer 783 IR spectrophotometer.

Production of L-DOPA by the mutant. The culture medium was clarified by centrifugation at 8000 r.p.m. for 10 min, followed by

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Abbreviation: L-DOPA, L-3,4-dihydroxyphenylalanine.

filtration through a 0.45 µm pore-size membrane filter. The filtrate was tested for the production of L-3,4 dihydroxyphenylalanine (L-DOPA) using the chemical method of Arnow (1937), high-performance liquid chromatography (HPLC) (Para *et al.*, 1984), and mass spectroscopy (MS) (Huizing *et al.*, 1985).

HPLC. Cell-free culture filtrates were acidified to pH 3.5 with 0.1 M-ammonium acetate, and 5 µl of this solution was injected on a reverse-phase column (Zorbax, ODS C18) through a rheodyne valve with a 20 µl sample loop connected to a Shimadzu liquid chromatograph (model LC-6A) and eluted with 0.01 M-ammonium acetate buffer (pH 3.5) at a flow rate of 1 ml min⁻¹, at 40 °C. The presence of L-DOPA was detected by measuring the absorbance at 280 nm. L-DOPA obtained from Sigma was used as reference standard.

MS. Cell-free culture filtrates showing the presence of a peak corresponding to standard L-DOPA peak by HPLC were partially purified following the procedure of Westerink & Mudler (1981), which is briefly as follows: a 1.5 ml aliquot of the culture filtrate was mixed with 50 µl perchloric acid and applied to a Sephadex G10 column (7 cm × 5 cm i.d.) and eluted with 3 × 1 ml 0.01 M-formic acid. The second 1 ml fraction eluted was found to contain L-DOPA (when analysed by HPLC) and this was lyophilized. The lyophilized fraction was reacted with 100 µl of trifluoroacetic anhydride at 50 °C for 15 min in a sealed tube to derivatize the L-DOPA. The standard L-DOPA was treated similarly. Both samples were then subjected to MS analysis using a JEOL-JMS DX 303 mass spectrometer under the control of a JEOL JMA-DA5000 data system, essentially according to the method of Huizing *et al.* (1985).

Results

Formation of the pigment

The dark-brown pigment was observed in the culture broth of *B. thuringiensis* H-14 mutant MB24 9 h after inoculation (Fig. 1). At this stage the vegetative growth of the organism had ceased. Microscopic examination of bacteria from this culture showed the presence of forespores, and a spore count indicated the presence of heat-resistant spores. Thereafter, the concentration of the pigment in the culture broth increased gradually and reached peak levels after 21 h. Parallel cultures of the parent strain B17 did not produce the pigment.

Effect of culture-medium composition on melanin production

All the media tested supported the production of the pigment by the mutant, giving colonies which were dark brown in colour, whereas those of the wild-type strain were creamy white. The cell mass of the mutant was also pigmented after 4–5 d of incubation. The intensity of the colour of the pigment was highest in potassium mineral salt (KMS) and casein/glycerol (CL) agar, followed by brain heart infusion, Penassay agar, Mueller–Hinton agar and nutrient agar (Table 1). This trait was tested for its stability on KMS agar and CL agar media and found to be stable even after 250 subcultures.

Identification of the pigment

Since the dark-brown pigment was indicative of a melanin (Ivins & Holmes, 1980) it was subjected to tests which are diagnostic for melanin. All the chemical tests

Fig. 1. Dynamics of biomass production (Δ), sporulation ($\square$) and pigment formation ($\circ$) during growth of *B. thuringiensis* H-14 mutant MB24 in 200 ml potassium mineral salts broth.

Table 1. Intensity of the pigment produced by *Bacillus thuringiensis* H-14 mutant MB24 grown on different agar media for 48 h at 30 °C

Media	Pigment intensity*
Nutrient agar	+
Mueller–Hinton agar	+
Penassay agar	++
Brain heart infusion agar	++
Casein/glycerol agar	++++
Potassium mineral salts agar	++++

* Visual observation.

Fig. 2. IR spectra of melanin from *B. thuringiensis* H-14 mutant MB24 (A) and authentic sample (B). Cultures were grown in casein/glycerol broth for 4 d.

Fig. 3. HPLC analysis of L-DOPA from an authentic sample (a) and culture broth of *B. thuringiensis* H-14 (b). Cultures were grown in potassium mineral salts broth.

were positive for melanin. The pigment was soluble in alkaline conditions (Aurstad & Dahle, 1972) and insoluble in organic solvents such as ether, chloroform and amyl alcohol (Shivaprasad & Page, 1989). It precipitated under acidic conditions (Bull, 1970; Ivins & Holmes, 1980). Addition of ferric chloride to the pigment solution resulted in the formation of a flocculant rusty-brown precipitate, and a mixture of ferrous sulphate and ferricyanide solutions caused a blue-green colour. Hydrogen peroxide bleached the dark-brown colour of the pigment to a colourless state (Bull, 1970). The IR spectra of the sample and the reference pigment were super-

Table 2. Effect of some amino acids and sodium ascorbate on the production of melanin by *Bacillus thuringiensis* H-14 mutant MB24

Cells were grown in minimal broth (Davis & Mingioli, 1950) containing 1% (w/v) casein for 3 d at 30 °C.

Addition to growth medium (0.1%)	Melanin concn (A_{520})†
L-Tyrosine	0.095 ± 0.003*
L-Phenylalanine	0.083 ± 0.005
L-Cysteine	0.082 ± 0.002
Arginine/HCl	0.000
Sodium ascorbate	0.000
Control	0.076 ± 0.002

* Value significantly different from other treatments (Duncan's multiple range test, $P < 0.05$, $n = 3$).

† A_{520} of the culture supernatant; mean ± SD.

imposable (Fig. 2) and showed absorbances at 2.9 μm (—OH stretch), 3.5 μm (—CH stretch), 5.9 μm (C=O stretch) and 6.1 μm (C=C stretch). The characteristics of the IR spectra of the pigment are similar to those reported in previous studies of melanins (Ellis & Griffiths, 1974).

Effect of amino acids and sodium ascorbate on pigment formation

Pigment formation during growth was enhanced in the presence of L-tyrosine in the culture medium compared to L-phenylalanine or L-cysteine (Table 2). When the mutant was grown in the presence of arginine/HCl or sodium ascorbate, pigment formation was suppressed.

Fig. 4. MS analysis of an authentic L-DOPA sample (a) and a partially purified sample from a culture of *B. thuringiensis* H-14 mutant MB24 (b). Cultures were grown in potassium mineral salts broth.

Production of L-DOPA by MB24

The culture broth of the mutant was tested for the presence of L-DOPA. When tested by the method of Arnov (1937) there was formation of a red colour, similar to that observed with authentic L-DOPA. The presence of L-DOPA was analysed further using HPLC after partial purification by chromatography on Sephadex G-10. The chromatogram of the sample showed the presence of a peak with the retention time of 5.3 min (Fig. 3), similar to that obtained with an authentic standard. Since catechol has been reported to act as a precursor for melanin (Shivaprasad & Page, 1989) its presence in the culture broth was checked by HPLC; it was absent (data not shown). The L-DOPA from the culture broth was purified further by preparative HPLC and subjected to MS. The spectrum obtained showed the presence of a molecular ion peak at 197, similar to that in the spectrum of an authentic standard (Fig. 4).

Detectable amounts of L-DOPA appeared in the cultures of the mutant when they reached the late-exponential phase of growth. The concentration of L-DOPA increased gradually to a peak level of $27.7 \mu\text{g l}^{-1}$ after 20 h culture when the bacteria were in decline phase, showing mature/lysing sporangia. Thereafter, the L-DOPA concentration decreased, with a simultaneous increase in the amount of the pigment.

Discussion

The *B. thuringiensis* H-14 mutant MB24 produces a dark-brown pigment in a range of culture media, during the stationary phase of growth, as reported for *Azospirillum brasilense* (Lakshmi & Neyra, 1987). Production of such a pigment by wild-type strains of *B. thuringiensis* and *B. cereus* has been reported previously by Aronson & Wermus (1965). However, these organisms produced pigment only when induced by the presence of D,L- β (3-hydroxyphenyl)alanine, an analogue of tyrosine. Mutations resulting in the constitutive production of this kind of pigment have been reported only in *Vibrio cholerae* (Ivins & Holmes, 1980).

The amino acids L-tyrosine, L-phenylalanine and L-cysteine have been reported to be precursors of melanins in other organisms (Raper, 1928; Protta, 1972; Swan, 1974). L-Tyrosine appears to be the precursor of L-DOPA in the case of the *B. thuringiensis* mutant as it enhanced the level of melanin production. Pomerantz & Murthy (1974) found that arginine/HCl inhibited the enzyme tyrosinase that catalysed the hydroxylation of L-tyrosine to L-DOPA and hence melanin. In the case of *B. thuringiensis* mutant MB24 also, arginine/HCl blocked the formation of melanin, indicating the involvement of a similar kind of enzyme in its production.

In the present study, the *B. thuringiensis* mutant

produced L-DOPA during early-stationary phase. Aronson & Vickers (1965) showed that *B. thuringiensis* can hydroxylate L-tyrosine to L-DOPA and this further confirms the present finding.

Analysis of the above results indicates that, in the *B. thuringiensis* mutant, L-tyrosine was hydroxylated to L-DOPA, catalysed by a tyrosinase, and ultimately converted to melanin. This type of pathway has been reported in some bacteria (Ivins & Holmes, 1980), actinomycetes (Kuster, 1976) and fungi (Bull, 1970; Ellis & Griffiths, 1974), although a different pathway, via catechol, was reported in *Azotobacter* sp. (Shivaprasad & Page, 1989).

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