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Induction of Melanin Biosynthesis in *Vibrio cholerae*

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Vibrio cholerae **synthesized the pigment melanin in response to specific physiological conditions that were stressful to the bacterium. Pigmentation was induced when *V. cholerae* was subjected to hyperosmotic stress in conjunction with elevated growth temperatures (above 30°C). The salt concentration tolerated by *V. cholerae* was lowered by additional abiotic factors such as acidic starting pH of the growth medium and limitation of organic nutrients. Although the amount of toxin detected in the culture supernatant decreased significantly in response to stressful culture conditions, no correlation between the physiological conditions that induced melanogenesis and expression of OmpU or cholera toxin was detected. Since conditions that induce melanin production in *V. cholerae* occur in both the aquatic environment and the human host, it is possible that melanogenesis has a specific function with respect to the survival of the bacterium in these habitats.**

Melanin, a dark brown-black pigment that occurs widely in plants and animals (17), may be grouped into three structural classes (23). The eumelanins and phaeomelanins occur primarily in animal species, and the allomelanins have been identified in microorganisms and plants (24). Bacteria for which melanin production has been described to occur include species of the genera *Aeromonas* (3), *Azotobacter* (30), *Mycobacterium* (27), *Micrococcus* (22), *Bacillus* (2), *Legionella* (4), *Streptomyces* (1), *Vibrio* (13, 26), *Rhizobium* (6), *Proteus* (35), and *Azospirillum* (28) as well as *Shewanella colwelliana* (11) and a periphytic marine bacterium designated strain 2-40 (14). Melanin is formed by the action of tyrosinase, a monooxygenase that has both cresolase and catecholase activities (15). The two enzymatic activities are coupled to mediate the conversion of tyrosinase via 3,4-dihydroxyphenylalanine to dopachrome, which is subsequently polymerized to melanin via a series of nonenzymatic reactions (26).

The genes involved in the virulence response of *Vibrio cholerae* have been extensively studied to produce an effective vaccine to combat this pathogen, which causes a severe diarrheal disease in humans (8, 9, 18). During the course of these studies, hypertoxinogenic mutants of *V. cholerae* 569B were isolated after treatment of the bacterium with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (18). One of these mutants, designated RM7, produced a brown pigment that was not phenotypically expressed in the wild-type strain, 569B (19). The pigment produced by RM7 and other independently isolated mutants was characterized and shown to be a melanin (13). The mutation that resulted in melanogenesis in *V. cholerae* RM7 and its derivatives was mapped to a locus linked to the *trp-1* marker on the chromosome and was not associated with the *htx* mutation responsible for hypertoxinogenicity (19). Although a number of additional phenotypic changes, such as alterations in toxinogenicity and motility, occurred in the pigmented *V. cholerae* mutants, it remains to be determined whether these are manifestations of pleiotropic effects of a single mutation or the result of multiple mutations after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (13).

Since it has been shown that expression of virulence-associated genes is modulated by the physical and nutritional properties of the growth media (8, 20), we investigated whether expression of the cryptic tyrosinase gene responsible for melanogenesis in *V. cholerae* RM7 could be induced in the wild-type strain in a similar fashion and, if so, what effect these environmental signals may have on expression of virulence determinants in this bacterium.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains included in this study were a non-O1 *V. cholerae* isolate (ATCC 2731), *V. cholerae* 569B (ATCC 25871), *V. cholerae* CA401 (ATCC 2277), *Vibrio vulnificus* ATCC 27562, and *Vibrio parahaemolyticus* ATCC 17802. All bacterial strains were maintained on 1% tryptone broth agar (Difco Laboratories, Detroit, Mich.) supplemented with 100 mM NaCl and stored in tryptone broth containing 15% glycerol at −70°C.

Induction of melanogenesis. Growth media (20 ml) were prepared in 125-ml Erlenmeyer flasks to ensure adequate aeration, which is necessary for tyrosinase activity. To test the effect of salinity, 1% tryptone broth was supplemented with NaCl at concentrations that ranged from 0.75 to 4%. Broth containing 0.75% NaCl supplemented with various concentrations of tryptone (1 to 0.016%) was used to investigate whether limitation of organic nutrients would induce pigmentation, and the effect of pH on melanogenesis was examined in 1% tryptone broth–0.9% NaCl–0.1 M phosphate buffer (pH 5.8 to 7.8). All growth media contained 5 mM L-tyrosine (Sigma Chemical Co., St. Louis, Mo.), which acted as a substrate for the tyrosinase enzyme responsible for melanin formation. Cultures were inoculated with 400 μl of a 5-ml overnight culture (1% tryptone broth, 0.6% NaCl; 30°C) and agitated at 350 rpm on a rotary shaker. Growth temperatures ranged from 5 to 37°C. Pigmentation was scored qualitatively by visual observation every 12 h. Data represent the results of three independently performed experiments.

Effect of glycinebetaine and L-proline on melanogenesis. Tryptone broth containing 3.5% NaCl and 5 mM L-tyrosine was supplemented with either glycinebetaine (Sigma) or

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L-proline (Sigma) at concentrations ranging from 5 to 25 mM. In subsequent experiments, 20 mM L-proline was added to the following media: 1% tryptone–1% NaCl–0.1 M phosphate buffer (pH 6.4), 0.125% tryptone–0.75% NaCl, and 0.125% tryptone–2.5% NaCl (all of the media were supplemented with 5 mM L-tyrosine). Growth was carried out at 30 or 37°C with rapid agitation. The cultures were scored for pigmentation as described above. Experiments were performed in triplicate.

Determination of OmpT and OmpU. *V. cholerae* 569B was propagated under conditions that either induced or had no effect on melanogenesis. Outer membrane proteins were isolated from these various cultures by urea extraction (16) and separated by electrophoresis through 12.5% polyacrylamide containing 0.4% sodium dodecyl sulfate. The proteins were visualized by staining with Coomassie brilliant blue R (Sigma) by a standard protocol (29). The two major outer membrane proteins, OmpT and OmpU, were quantified by determining the densities of the protein bands; the results are expressed as percentages after Gaussian integration of the peaks.

Determination of cholera toxin. Cholera toxin in the supernatants of the above cultures was detected by a modification of the GM_1 ganglioside enzyme-linked immunosorbent assay technique of Holmgren (12). GM_1 receptors (Sigma) were suspended in 0.1 M Tris buffer (pH 7.5) at a final concentration of 2 μg ml^{-1}. Aliquots (100 μl per well) of this suspension were added to a 96-well assay plate (Corning low binding easy wash; Corning Glass Works, Corning, N.Y.), which was incubated overnight at 4°C. After nonbound material was removed by rinsing with wash buffer (0.05% Tween 20 in phosphate-buffered saline [137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4]), fivefold serial dilutions of the culture supernatants were prepared in duplicate in dilution buffer (5% Carnation nonfat dry milk in phosphate-buffered saline) and added to the wells of the assay plate, which was then incubated at 25°C for 24 h. Dilution buffer (200 μl) was added to each well, and the plate was incubated for 60 min at 25°C to prevent antibody binding to exposed polystyrene surfaces. Polyclonal antibodies to cholera toxin were added to the assay plate, which was incubated at 37°C for 1 h. The bound antibody was detected with affinity-purified peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) and the developing substrate *o*-phenylenediamine (Sigma), freshly prepared at a concentration of 0.4 mg ml^{-1} in *o*-phenylenediamine substrate buffer (14.7 g of trisodium citrate per liter, 14.2 g of Na_2HPO_4 per liter [pH 5.0]) containing 0.012% hydrogen peroxide (Sigma), by determining the A_{492}. The number of viable cells in each bacterial culture at the time of sampling was determined by plating appropriate dilutions of the cell suspension on Luria agar. Cholera toxin concentrations were determined by extrapolation from a standard curve of known concentrations of commercial cholera toxin (Sigma) versus A_{492} and standardized according to the viable cell count.

RESULTS

Effect of temperature and salinity. Temperatures between 5 and 25°C failed to induce melanogenesis in *V. cholerae* 569B when the microorganism was grown in tryptone broth with NaCl concentrations of 0 to 4%. However, growth temperatures of 30°C and above, in conjunction with elevated salinity of the growth medium, induced pigmentation. *V. cholerae* 569B produced pigment after 2 days of growth at 30°C in the presence of 4% NaCl, whereas cultures propagated at 37°C produced pigment after 1 day when exposed to a salt concentration of 2%.

TABLE 1. Effect of nutrient limitation on melanogenesis in *V. cholerae* 569B grown at 30°C

% Salinity	Day	Pigmentation of cells grown on the following % of tryptone						
		1.0	0.5	0.25	0.125	0.063	0.031	0.016
0.75	3	−	−	−	−	−	+	+
	4	−	−	+	+	+	+	+
	5	−	+	+	+	+	+	+
2.5	3	−	−	−	+	+	+	+
	4	−	−	+	+	+	+	+
	5	−	+	+	+	+	+	+

Effect of the starting pH of the growth medium. The starting pH of the tryptone broth had no effect on pigmentation when cells were grown at temperatures ranging from 5 to 30°C. However, melanin production occurred after 3 days when *V. cholerae* 569B was grown at 37°C in culture media in which the initial pH ranged from 5.8 to 6.6. Pigmentation was not induced in bacterial cultures grown at 37°C when the starting pH of the medium was greater than 6.6.

Effect of nutrient limitation. Nutrient-rich growth media with a salinity of 0.75% NaCl did not induce melanin synthesis at 30 or 37°C. However, *V. cholerae* 569B produced pigment when grown at 30°C in media in which the concentration of tryptone had been reduced (Table 1). The onset of pigmentation occurred earlier in media that contained lower nutrient concentrations, whereas an increase in the NaCl concentration from 0.75% to 2.5% resulted in melanin production in media containing a fourfold-greater concentration of tryptone. Nutrient deprivation failed to elicit pigmentation when *V. cholerae* 569B was grown at temperatures below 30°C.

Effect of glycinebetaine and L-proline. Since the salinity of the growth medium seemed to be important with respect to induction of pigmentation, we investigated whether melanization occurs in *V. cholerae* 569B in response to osmotic stress. The osmoprotectants glycinebetaine and L-proline were tested for their ability to suppress pigmentation when the wild-type strain was grown under pigment-inducing conditions. Glycinebetaine delayed pigmentation for approximately 36 h when the wild-type strain was grown in the presence of 3.5% NaCl at 37°C (Table 2). Similarly, 5 to 15 mM L-proline delayed melanogenesis for 1 to 2 days, whereas L-proline concentrations of 20 mM and above inhibited pigmentation in *V. cholerae* 569B (Table 2). Since L-proline clearly prevented pigmentation in *V. cholerae* 569B, its effect on pigmentation in cells grown under various melanogenic conditions was investigated further (Table 3). Thus, the addition of 20 mM L-proline to cultures propagated in media that normally induce pigmentation in *V. cholerae* 569B prevented the formation of melanin.

Effect of melanogenic physiological conditions on expression of virulence determinants. The ratio of the two major outer membrane proteins, OmpT (40 kDa) and OmpU (38 kDa), remained relatively constant with respect to the culture conditions tested (Table 4). Although the level of cholera toxin produced and secreted by *V. cholerae* 569B that had been subjected to various stresses was significantly lower than that of the unstressed control, no relationship between

TABLE 2. Effect of osmolytes on melanogenesis in *V. cholerae* 569B

Osmolyte and concn (mM)	Pigmentation on day:		
	1	2	3
Glycinebetaine			
0	+	+	+
5	−	+	+
10	−	+	+
15	−	+	+
20	−	+	+
25	−	+	+
Proline			
0	+	+	+
5	−	+	+
10	−	+	+
15	−	−	+
20	−	−	−
25	−	−	−

TABLE 4. Expression of OmpT and OmpU in *V. cholerae* 569B grown under various culture conditions

Culture conditions	Day	% OmpT[a]	% OmpU	Pigmentation
1% tryptone, 30°C	1	67 (2.1)	33	−
pH 6.4, 30°C	2	56 (2.0)	44	−
	4	63 (2.7)	37	−
pH 6.4, 37°C	2	57 (6.7)	43	−
	4	65 (2.4)	35	+
0.125% tryptone, 30°C	2	75 (3.1)	25	−
	4	61 (9.4)	39	+
0.125% tryptone, 37°C	2	69 (2.3)	31	−
	4	58 (5.5)	42	+

[a] The data represent the averages of four experiments, with the standard errors shown in parentheses.

the physiological conditions that induce pigmentation and toxin production was observed (Table 5).

Induction of pigmentation in other *Vibrio* strains. *V. cholerae* 569B, CA401, and ATCC 2731 produced melanin in response to the same physiological conditions: a growth medium starting pH of 6.4 at 37°C and nutrient limitation (0.125% tryptone) at both 30 and 37°C. *V. vulnificus* produced pigment only when grown in 0.125% tryptone broth supplemented with 0.6% NaCl at 37°C, whereas *V. parahaemolyticus* failed to synthesize melanin in response to any of the conditions tested.

DISCUSSION

A hypertoxic *V. cholerae* mutant produced a melanin pigment, indicating that a silent tyrosinase gene was possibly present in the nonpigmented wild-type strain, 569B (13, 19). Since expression of the melanin phenotype in the mutant strain was influenced by the nature of the growth medium (13), we investigated whether pigmentation could be elicited in strain 569B in a similar fashion. Culture conditions such as temperature, pH, and salinity were varied, since these have been reported to affect expression of a number of virulence determinants in *V. cholerae* 569B (8, 20).

Melanogenesis was induced by a variety of physiological conditions that are stressful to *V. cholerae* 569B. Growth temperatures of 30°C induced melanogenesis at salinities of 4%, whereas *V. cholerae* 569B propagated at 37°C produced pigment at lower salinities. In addition, *V. cholerae* 569B produced pigment when the starting pH of the medium was acidic, and also in response to nutrient limitation, when grown at elevated temperatures. These conditions elicited a similar response in the other two *V. cholerae* strains examined.

Hyperosmotic stress seems to be the primary environmental signal that induces melanogenesis in *V. cholerae* 569B; pigmentation did not occur when the bacterium was grown under melanogenic culture conditions in the presence of the osmoprotectants glycinebetaine and L-proline. *V. cholerae* tolerates salinities in the range of 0 to 3% NaCl (5); 2.5% salt is optimal for growth (32, 33). However, *V. cholerae* is more susceptible to low and high salt concentrations when subjected to starvation or extreme temperatures (32–34). Thus, increased salinity induced pigmentation in *V. cholerae* at temperatures of 30°C and above, whereas pigmentation occurred at lower salt concentrations when the bacterium was subjected to additional stressful factors such as low organic nutrient concentrations and acidic pH of the growth medium. The observation that pigmentation in *V. vulnificus* was induced only when the bacterium was subjected to nutrient limitation and increased temperature at 0.6% NaCl, whereas *V. parahaemolyticus* did not produce pigment in response to any of the conditions tested, may be explained by the fact that these microorganisms tolerate salt concentrations of 6 and 8%, respectively (5). In other words, a great deal of stress (nutrient limitation in conjunction with an elevated growth temperature) was necessary to induce *V. vulnificus* to produce pigment at a salt concentration of 0.6%, because this salinity is not normally stressful to this

TABLE 3. Effect of L-proline on melanogenesis in *V. cholerae* 569B

Culture condition	Pigmentation	
	Control	20 mM proline[a]
1% tryptone (pH 6.4), 37°C	+	−
0.125% tryptone–0.75% NaCl, 30°C	+	−
0.125% tryptone–0.75% NaCl, 37°C	+	−
0.125% tryptone–2.5% NaCl, 30°C	+	−
0.125% tryptone–2.5% NaCl, 37°C	+	−[b]

[a] Proline was added to cultures.
[b] Pigmented after 4 days of incubation.

TABLE 5. Effect of various culture conditions on synthesis of cholera toxin

Culture conditions	Toxin concn[a] (ng/10^7 cells/ml)	Pigmentation
1% tryptone, 30°C	54.0	−
pH 6.4, 30°C	15.0	−
pH 6.4, 37°C	ND	+
0.125% tryptone, 30°C	6.4	+
0.125% tryptone, 37°C	2.6	+

[a] Determined from a standard curve of known concentrations of cholera toxin with the average absorbance of a duplicate culture supernatant dilution series and standardized according to the number of viable cells. ND, not detectable.

halophile. However, it is possible that insufficient stress was applied to render *V. parahaemolyticus* sensitive to 0.6% NaCl, since this microorganism is more halotolerant than *V. vulnificus*. It is likely that *V. parahaemolyticus* will produce pigment at higher salinities when subjected to additional environmental stresses such as elevated temperature and starvation. This implies that melanogenesis occurs in response to a specific combination of environmental signals that are characteristic of specific habitats in which these microorganisms may occur.

Although glycinebetaine and L-proline have been reported not to function as osmoprotectants in *V. cholerae* in terms of stimulation of growth in media of high osmolarity (7), the compounds delayed and prevented melanogenesis, respectively. This may indicate that these compounds provide *V. cholerae* with some form of protection against hyperosmotic stress.

To investigate whether the physiological conditions that induce melanogenesis in *V. cholerae* affect the virulence of this bacterium, expression of OmpU and cholera toxin was examined under melanin-inducing and noninducing growth conditions. The ratio of OmpT to OmpU (associated with the aquatic environment and the virulence response, respectively [20]) remained relatively constant when the bacterium was propagated under conditions that induced pigmentation compared to those that did not elicit the phenotype. Although production of cholera toxin decreased when *V. cholerae* 569B was grown under stressful conditions, no relationship between the physiological conditions that induce melanogenesis and production of cholera toxin was observed.

Our inability to detect a correlation between culture conditions that induce melanin production and expression of OmpU and cholera toxin does not entirely eliminate a role for melanogenesis in *V. cholerae* pathogenesis. Parsot and Mekalanos (25) have shown that toxin production is significantly decreased at 37°C, which is the temperature encountered by the bacterium once it invades the human host, and yet the toxin is the causal agent of the severe symptoms experienced by cholera sufferers. A complex series of events, mediated by a regulatory cascade system, has been postulated to occur upon infection of a human host (8, 9, 25). It is possible that melanin functions during an early stage of the virulence response, whereas OmpU and cholera toxin are expressed at a later stage of infection. The combination of the low pH of the stomach, increased temperature, and limited nutrients may cause the bacterium to become susceptible to the osmolarity of the mucosal lining of the upper small intestine, which is equivalent to 0.9% NaCl (20). The onset of hyperosmotic stress may then induce *V. cholerae* to synthesize melanin. We have shown that melanogenesis is induced in vitro by physiological conditions that reflect those that occur in the human host. The fact that melanogenesis is inhibited by the osmolyte L-proline when *V. cholerae* is grown at 0.9% NaCl and 37°C in medium that is either nutritionally limited or has an initial pH of 6.4 supports the conclusion that the bacterium is osmotically stressed when it is subjected to the extreme physiological conditions encountered in the human host. The ability to synthesize melanin at the colonization stage of the virulence response would be advantageous with respect to the pathogenicity of *V. cholerae*, since the free radical-scavenging properties of melanin (31) may enable the invading bacteria to endure the severe oxidative stress associated with macrophage phagocytosis (10). Melanogenesis in Na^+-dependent strains of *Azotobacter chroococcum* has been shown to be suppressed in the presence of charcoal (free radical trap) and benzoic acid (free radical scavenger), indicating a relationship between the presence of toxic oxygen species and pigmentation (31). Once the walls of the upper small intestine have been colonized by *V. cholerae*, an as yet unknown stimulus may then induce synthesis of cholera toxin.

Alternatively, melanogenesis may enhance the survival of *V. cholerae* in estuarine environments during the summer months, when the water temperature rises and the salinity increases because of evaporation. Melanin has been reported to possess cation-exchange properties, as demonstrated by its capacity to absorb Na^+ and K^+ ions (36). Thus, the polymer may act to prevent cellular dehydration by sequestering compatible solutes from the environment. In addition, hydroxyl radicals occur at concentrations of 1.1×10^{-18} and 12×10^{-18} M in open ocean surface water and coastal surface water, respectively (21). A second possible role for melanogenesis in the aquatic environment could be to alleviate oxidative stress by scavenging free radicals, which are abundant during the summer months when UV B irradiation increases.

On the basis of the results presented here, we conclude that melanogenesis is either an integral component of the virulence response of *V. cholerae* or important ecologically in terms of the survival of *V. cholerae* in the aquatic environment; it is possible that melanogenesis is important in both habitats. Whether the melanin polymer has a specific function related to the above, or whether it assumes a more generalized role in terms of overall osmoregulation, remains to be elucidated.

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