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NOTES

Homogentisic Acid Is the Primary Precursor of Melanin Synthesis in *Vibrio cholerae*, a *Hyphomonas* Strain, and *Shewanella colwelliana*

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The enzyme *p*-hydroxyphenylpyruvate hydroxylase (HPPH) is involved in pigmentation (pyomelanin) via homogentisic acid (HGA). Pyomelanin formation is correlated with HGA production and expression of HPPH in three disparate marine species: *Vibrio cholerae*, a *Hyphomonas* strain, and *Shewanella colwelliana*. Induction of pigmentation in *V. cholerae* 569B by nutrient limitation also correlated with production of HGA.

A number of bacterial species have been reported to produce melanin (1, 2, 11, 22, 26, 29, 30, 34); however, very few of these pigmentation systems have been molecularly defined (3, 12, 13, 15, 17, 33). The best-understood melanization pathway is the classic Mason-Raper pathway (31), in which tyrosinases yield the melanin intermediate dihydroxyphenylalanine (DOPA). Other pathways, producing other types of melanin, have also been reported (6, 14, 16, 18, 36, 40). For example, pyomelanin is derived from the catabolism of tyrosine via *p*-hydroxyphenylpyruvate (HPP) and homogentisic acid (HGA). The enzyme catalyzing this reaction is *p*-hydroxyphenylpyruvate hydroxylase (HPPH; EC 1.13.11.27), which was first recognized and most intensively studied in higher organisms (8, 9, 21, 23, 32). When HPPH is not rate limiting or when downstream enzymes in this pathway (e.g., homogentisic acid oxidase) are dysfunctional (25), HGA accumulates and polymerizes to the water-soluble red-brown pigment pyomelanin or alkapton (25). Alkaptonuria, a rare disease in humans who lack HGA oxidase, is diagnosed by HGA and alkapton accumulation in the urine (25). Among bacteria, pyomelanin was demonstrated in *Pseudomonas aeruginosa*, and the amino acid sequence of its HPPH was determined, but the gene has not been cloned (24, 28, 33, 40).

We have been studying melanogenesis in *Shewanella colwelliana*, a marine bacterium, correlating it with recruitment of the oyster *Crassostrea virginica* (10, 38, 39). The *S. colwelliana melA* gene, putatively coding for HPPH (6), has been cloned, sequenced, and expressed in *Escherichia coli* (12, 13). Homogentisic acid was unambiguously identified as the MelA enzymatic reaction product (6). The predicted amino acid sequence of MelA has significant sequence homology with HPPH from *Pseudomonas* sp. strain P.J.874 (6, 33).

While wild-type *Vibrio cholerae* 569B is not normally melanogenic in culture, its hypertoxic mutant (strain HTX-3) was reported to synthesize a phaeomelanin (16). Subsequently, Coyne and Al-Harthi (7) reported that, under stress, *V. cholerae* 569B could be induced to synthesize a eumelanin. However, to date, there is no information on the enzymes or genes involved in *V. cholerae* melanization.

Here we show that pigmenting *V. cholerae* and a pigmenting marine *Hyphomonas* sp. produce HGA and express proteins which cross-react with antibodies to *S. colwelliana* HPPH. In contrast, nonpigmenting cultures produce neither HGA nor HPPH, suggesting that the melanin produced by *V. cholerae* and the *Hyphomonas* sp. is pyomelanin.

In this study, matched pairs of constitutively pigmenting and nonpigmenting strains of three species of marine bacteria were used. The pigmenting strains were *V. cholerae* HTX-3 (16), *S. colwelliana* D (35, 38), and *Hyphomonas* sp. strain MHS-3 PM1 (18), and the nonpigmenting strains were *V. cholerae* 569B (ATCC 25871); *S. colwelliana* W (39); *S. colwelliana* C75, a *melA* null mutant (13); and *Hyphomonas* sp. strain MHS-3 (39). These marine bacteria were grown in marine broth (MB) or MB supplemented with 4 mM tyrosine (MBT) at 25°C with vigorous aeration; *V. cholerae* strains were also cultured at 37°C. The nonpigmenting strain *E. coli* JM101(pUC19) and the pigmenting strain *E. coli* JM101 carrying the *S. colwelliana melA* gene on plasmid pMC3B (11) were grown at 37°C in Luria-Bertani medium supplemented with isopropylthio-β-D-galactoside (IPTG; 100 μg/ml), ampicillin (100 μg/ml), and 4 mM tyrosine.

Pyomelanin production, estimated visually, and HGA synthesis, determined electrochemically by high-pressure liquid chromatography (HPLC; C_{18} reverse-phase column; mobile phase, 7.5% acetonitrile in 10 mM monochloroacetic acid, 1.3 mM EDTA, and 1.3 mM sodium octyl sulfate [pH 2.6] [6]) were ascertained during time course experiments on MB or MBT medium. *V. cholerae* 569B was examined for pyomelanin and HGA synthesis under nutrient-limited conditions (0.125% tryptone, 2.5% salt, and pH 6.4) at 25 and 37°C. The presence of HPPH activity was determined from cell lysates and spent medium by using tyrosine as a substrate, as described previously (6, 11).

Parallel aliquots of cell lysates were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting) on nitrocellulose. Blots were probed with a MelA-specific polyclonal anti-

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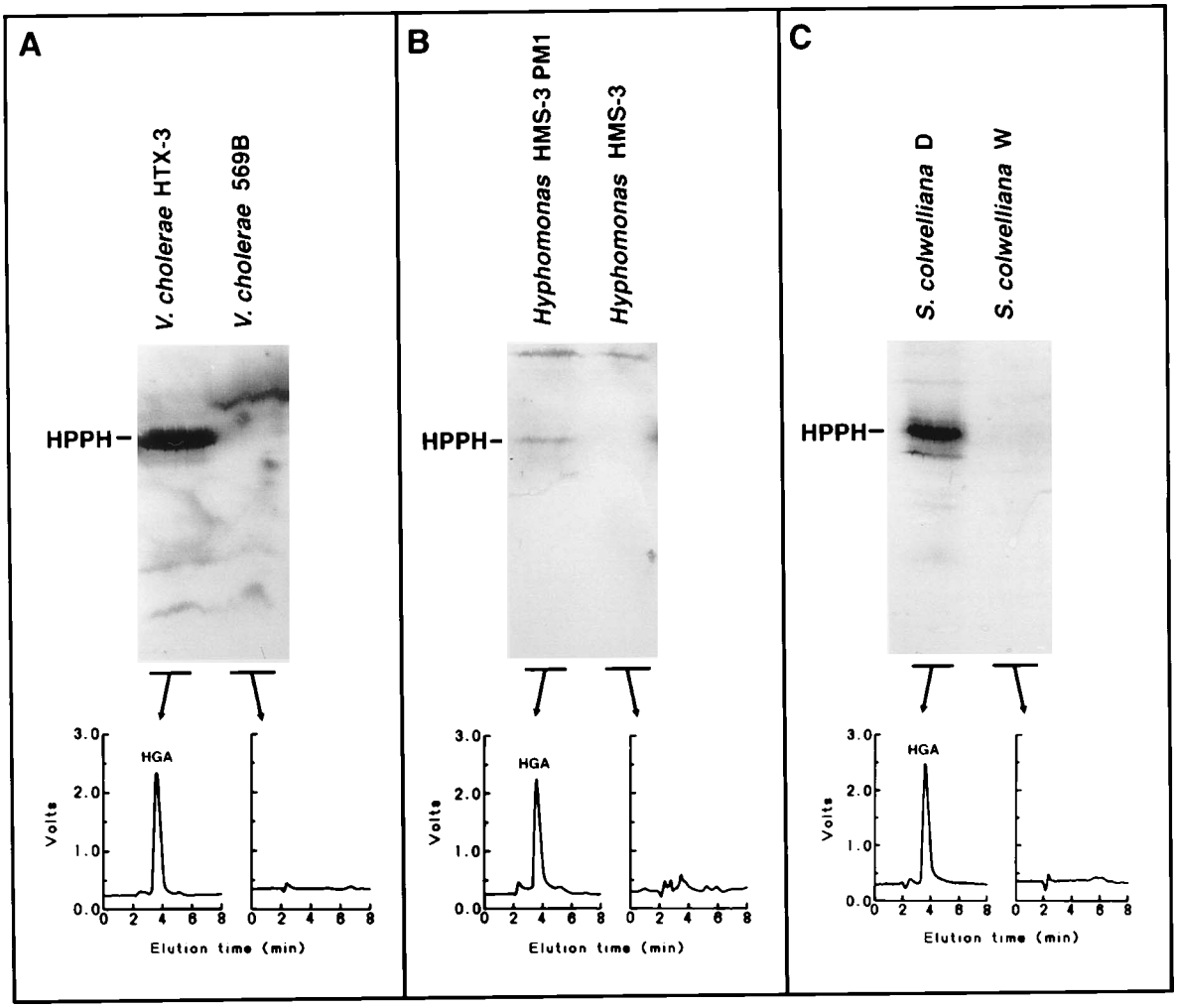

FIG. 1. Comparison of HPPH and HGA production in hyperpigmenting and nonpigmenting bacterial strains. Stationary-phase cultures of each strain were harvested. Samples of cells were separated by SDS-PAGE (12% acrylamide), electroblotted onto a nitrocellulose membrane, and probed for HPPH (41k Da) by immunoblotting with a purified antibody to the MelA-LacZ fusion protein from *S. colwelliana* D. The corresponding supernatants were assayed for HGA by HPLC with electrochemical detection; HGA eluted at 3.9 min. Chromatographs are shown under the appropriate lane from the Western blots.

serum generated against a MelA-LacZ affinity-purified fusion protein (11–13). This antibody preparation was partially purified by absorbing antibodies with LacZ.

During growth, the constitutively hyperpigmenting variants *V. cholerae* HTX-3, *S. colwelliana* D, and *Hyphomonas* sp. strain MHS-3 PMI produced increasing amounts of red-brown water-soluble melanin with time; pigmentation was augmented by the addition of tyrosine. *E. coli* JM101(pMC3B) produced a pigment of similar appearance. In contrast, *V. cholerae* 569B, the *S. colwelliana* C75 *melA* null mutant, *S. colwelliana* W, *Hyphomonas* sp. strain MHS-3, and *E. coli* JM101(pUC19) did not produce visible pigment at any time, consistent with previous observations (11–13). Of the nonconstitutively pigmenting strains, only *V. cholerae* 569B produced pigment, and only under nutrient-limited conditions (7). Both *V. cholerae* HTX-3 and *V. cholerae* 569B produced red-brown water-soluble pigment, not phaeomelanin- or eumelanin-type pigments, as previously reported (7, 16).

Pigment production consistently correlated with the detection of HGA in the spent medium and cell lysates of all pigmenting strains, including *E. coli* JM101(pMC3B). Homogentisic acid was not detected in the medium or lysates of nonpigmenting strains. Interestingly, *V. cholerae* 569B also produced HGA in the spent medium, but only under the same stress conditions which induced pigmentation. In fact, the more rapid onset of pigmentation at 37°C than at 25°C was paralleled by an earlier detection of HGA. Lysates of *S. colwelliana* D and *E. coli* JM101(pMC3B) produced HGA when HPP but not *p*-hydroxyphenylacetate was substituted for tyrosine, consistent with the conventional pathway of tyrosine degradation in eucaryotes (data not shown). This implies the presence of tyrosine transaminase in these organisms, catalyzing the conversion of tyrosine to HPP.

Purified antibody to the MelA-LacZ fusion protein from *S. colwelliana* cross-reacted with proteins synthesized by *V. cholerae* HTX-3, *Hyphomonas* sp. strain MHS-3 PMI, and *S. colwelliana* D, all of which were pigmenting when harvested (Fig. 1). The antibody did not visibly detect HPPH in the corresponding nonpigmenting strains. The 44-kDa protein in *V. cholerae* 569B (Fig. 1A) and the 52-kDa protein in *Hyphomonas* sp. strain MHS-3 (Fig. 1B) may be cross-reacting with incompletely absorbed antibodies in the partially purified preparation. Among the controls, there was a strong reaction to the *E. coli* strain carrying the cloned *melA* gene on pMC3B; however, no detectable band was observed with either *E. coli* JM101(pUC19) or the *melA* null mutant *S. colwelliana* C75

(data not shown). In all cases, immunodetectable HPPH correlated with detection of HGA in the spent medium (Fig. 1).

The hypertoxic mutant of *V. cholerae*, strain HTX-3, has been reported to synthesize phaeomelanin, a red DOPA-melanin incorporating more cysteine than is incorporated into eumelanin (16). It was also reported that *V. cholerae* 569B produced eumelanin (DOPA based) when subjected to stress such as elevated temperature, i.e., physiological conditions that reflect those of human hosts (7). In our hands, both *V. cholerae* strains produced pyomelanin (alkapton), as determined by the solubility of the pigment, the presumptive immunoblot detection of HPPH correlating with pigmentation, and, most importantly, the identification of HGA but not DOPA (6, 11–13) in spent medium. These results do not unequivocally rule out the possibility that a DOPA-based melanin was also synthesized in very low quantities.

Pyomelanin production from tyrosine via the HGA pathway has been reported for several *Pseudomonas* strains (24, 28, 40) and the marine bacterium *S. colwelliana* D (6). Additionally, HGA has been described as a catabolite of phenolic metabolism in other procaryotes (4). To our knowledge, this is the first demonstration of the biosynthesis of pyomelanin in the marine bacteria *V. cholerae* HTX-3 and the *Hyphomonas* sp. In these two bacteria, as well as in *S. colwelliana* D, the mechanism of pyomelanin synthesis would be the derepression of HPPH rather than a mutated downstream enzyme, as suggested by the detection of the enzyme in Western blots of pigmenting bacteria but not in corresponding nonpigmenting strains. The molecular genetic mechanism of constitutive derepression has been determined for *S. colwelliana* D (35).

Whether mutations leading to the constitutive hyperproduction of HPPH or the derepression of HPPH by environmental stress are artifactual or occur in nature is not known. However, a number of functions have been ascribed to bacterial melanins and their precursors, including induction of invertebrate set (41), invasiveness (37), virulence (19), biofilm adhesiveness (20), resistance to metals (27), and inhibition of polysaccharases (5).

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