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Isolation and Characterization of Strain MMB-1 (CECT 4803), a Novel Melanogenic Marine Bacterium

FRANCISCO SOLANO,[1] ENCARNACIÓN GARCÍA,[2] ENCARNACIÓN PÉREZ DE EGEA,[1] AND ANTONIO SANCHEZ-AMAT[2]*

Department of Biochemistry and Molecular Biology B[1] and Department of Genetics and Microbiology,[2] University of Murcia, 30100 Murcia, Spain

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A novel marine melanogenic bacterium, strain MMB-1, was isolated from the Mediterranean Sea. The taxonomic characterization of this strain indicated that it belongs to the genus *Alteromonas*. Under in vivo conditions, L-tyrosine was the specific monophenolic precursor for melanin synthesis. This bacterium contained all types of activities associated with polyphenol oxidases (PPOs), cresolase (EC 1.18.14.1), catecholase (EC 1.10.3.1), and laccase (EC 1.10.3.2). These activities were due to the presence of two different PPOs. The first one showed all the enzymatic activities, but it was not involved in melanogenesis in vivo, since amelanogenic mutant strains obtained by nitrosoguanidine treatment contained levels of this PPO similar to that of the wild-type MMB-1 strain. The second PPO showed cresolase and catecholase activities but no laccase, and it was involved in melanogenesis, since this enzyme was lost in amelanogenic mutant strains. This PPO was strongly activated by sodium dodecyl sulfate below the critical micelle concentration, and it is a tyrosinase-like enzyme showing a lag period in its tyrosine hydroxylase activity that could be avoided by small amounts of L-dopa. This is the first report of a bacterium that contains two PPOs and also the first report of a pluripotent PPO showing all types of oxidase activities. The bacterium and the pluripotent PPO may be useful models for exploring the roles of PPOs in cellular physiology, aside from melanin formation. On the other hand, the high oxidizing capacity of the PPO for a wide range of substrates could make possible its application in phenolic biotransformations, food processing, or the cosmetic industry, where fungal and plant PPOs are being used.

Melanins are dark-colored polyphenolic pigments synthesized by different organisms throughout the phylogenetic scale, from bacteria to mammals. Melanins are involved in defensive functions against oxidants, free radicals, and UV radiation, although the exact mechanisms for these roles are still largely unknown. In higher organisms, melanin is made by using L-tyrosine as a precursor, and the key enzyme is tyrosinase (EC 1.14.18.1). This enzyme catalyzes the first two reactions in the Raper-Mason pathway: *o*-hydroxylation of L-tyrosine (cresolase activity) into 3,4-L-dihydroxyphenylalanine (L-dopa) and the subsequent oxidation of that *o*-diphenol to yield L-dopaquinone (catecholase activity) (27). After formation of this *o*-quinone, the pathway can proceed spontaneously, since this compound is not stable and undergoes an intramolecular cycling yielding L-dopachrome, a well-known red-orange intermediate of melanin synthesis. Then L-dopachrome decarboxylation yields dihydroxyindole, and further rearrangements and polymerization of these units lead to melanin (22).

Tyrosinase is a copper protein that belongs to the group of polyphenol oxidases (PPOs). The other important copper enzyme in this group is laccase (EC 1.10.3.2). This enzyme was first described for the lacquer tree (39). Later, it was found in numerous fungi (35) and quite recently has been found in one bacterium (11). Laccase shows more affinity for the oxidation of *p*-diphenols than *o*-diphenols, but in fact tyrosinase and laccase are able to oxidize an overlapping range of diphenolic compounds. In spite of that, these enzymes have been traditionally differentiated on the basis of substrate specificity and sensitivity to specific inhibitors (36). Concerning substrate specificity, the most important differences between the two types of enzymes are the facts that only tyrosinase shows cresolase activity and the capacity to oxidize L-tyrosine (27) and only laccase is able to oxidize some bulky aromatic chromophores such as syringaldazine (7, 11, 35).

Fungal PPOs, particularly laccases, have been intensively studied. They have been related to a variety of functions concerning sexual differentiation, pigmentation of fruiting bodies, lignolysis, detoxification, and others (35). In recent years, fungal laccases have received a lot of attention because of their high capacity for oxidizing aromatic compounds. This feature makes laccases very suitable for some biotechnological applications, such as biodegradation of xenobiotic compounds (4) and biopulping in the paper industry (9). Similar applications have been proposed for tyrosinases, for example, the selective removal of contaminant by-products in industrial fermentation processes (26).

Tyrosinase and laccase are expressed simultaneously in some fungi (14). Furthermore, different isozymes of these PPOs are present in numerous species (4, 7, 9, 38). Since all these enzymes are able to oxidize several diphenols to yield pigments, their involvement in melanin formation has been frequently proposed. However, the functions of each enzyme have never been well delimited. Their roles in cell physiology and the advantages of expressing enzymes which are so closely related are not well understood.

Bacteria synthesize melanin pigments from monophenols basically through two pathways (30). The first is the formation of *o*-diphenols mediated by cresolase activity, and the second is the oxidation of intermediates of monophenol catabolism, such as homogentisate or other *p*-diphenols. The usual monophenol occurring in the cell is the amino acid L-tyrosine, so this is the main precursor of melanin pigments. As it is assumed that L-tyrosine is not a substrate of laccases (11, 35, 36), melanogenesis is determined by tyrosinase activity rather than laccase

* Corresponding author. Phone: (34) 68 307100. Fax: (34) 68 363963. E-mail: antonio@fcu.um.es.

activity. Tyrosinases occur more frequently than laccases in melanogenic bacteria.

The enzymes involved and the nature of melanin precursors in prokaryotic cells are not well known (23). One reason for this could be the small number of melanogenic strains which have been well characterized. Briefly, tyrosinase activity has been clearly shown in four strains, a *Streptomyces* sp. (21), a *Rhizobium* sp. (24), *Vibrio tyrosinaticus* (28), and the marine bacterium strain 2-40 (18). *Azospirillum lipoferum* is the only strain in which the involvement of laccase in melanization has been demonstrated (8). So far, there are no reports of any bacteria containing both tyrosinase and laccase activities. Other known melanogenic bacteria, such as *Vibrio cholerae* (29) and *Shewanella colwelliana* (5, 19, 30), are examples of microorganisms which produce melanin pigments by an alternative route, the excretion and polymerization of homogentisate (pyomelanin).

In this paper, we present the characterization of a novel melanogenic bacterium, strain MMB-1, isolated from the Mediterranean Sea. We have established the taxonomic classification of this strain, characterized its pathway of melanin synthesis, and studied some properties of its PPO system. This strain contains two PPOs, a tyrosinase-like PPO and another PPO showing all the activities of tyrosinase and laccase. The latter enzyme is shown to be a novel multifunctional PPO having cresolase, catecholase, and laccase activities.

MATERIALS AND METHODS

Strains. Strain MMB-1 was detected by M. Jansa during her bacterial sampling experiments with seawater from the Mediterranean coast of southeastern Spain. She kindly provided us this strain as well as other bacterial colonies displaying dark pigmentation when cultured in Marine Agar 2216 (Difco). For some experiments concerning taxonomy, *Vibrio anguillarum* CECT 552 and strain 2-40 (ATCC 43961) were used as controls. The genus of the latter strain is uncertain, but it shares many taxonomic characteristics with the genus *Alteromonas* (1).

Morphology. Strain MMB-1 was repeatedly subcultured in Marine Agar 2216 until a pure culture was obtained. Gram staining was performed according to standard protocols. For electron microscopy, cells were grown in Marine Broth 2216 (Difco). One drop of the culture was deposited on Formvar-coated grids and stained for 30 s with a 1% solution of phosphotungstic acid in distilled water (pH 6.7).

Physiological characterization. In order to test whether strain MMB-1 was able to grow anaerobically, Marine Broth 2216 and F1 medium (10) solidified with 0.3% agar were used. For each medium, two tubes were prepared and inoculated with the strain. Immediately, one of the tubes was covered with Parafilm, and the tubes were incubated at 25°C.

Accumulation of polyhydroxybutyrate (PHB), Na^+ requirements, luminescence, denitrification, and production of nitrites from nitrates were assayed according to the protocols of Gauthier and Breittmayer (10). Several extracellular enzymatic activities were also tested. Gelatinase activity was tested in Marine Broth 2216 supplemented with 12% gelatin. Tubes with 10 ml of this medium were inoculated and incubated for 6 days at 25°C. Gelatin hydrolysis was established by comparing the solidification of the test tube contents in a refrigerator against that of an uninoculated control (34). Amylase activity was tested in Marine Agar 2216 and YEA medium (10) supplemented with 2 g of starch per liter. Starch hydrolysis was revealed by using Lugol's iodine solution. To detect lipase activity, 10 g of Tween 80 per liter was added to Marine Agar 2216. Hydrolysis of Tween 80 was detected by the appearance of a precipitated halo of calcium oleate. A cytochrome *c* oxidase test was performed by two methods to confirm the results. In the first method, a drop of the oxidase reagent (1% tetramethyl-*p*-phenylenediamine) was deposited onto filter paper, and an isolated colony was streaked onto it (34). In the second method, 1 ml of a culture in Marine Broth 2216 was permeabilized by toluene, and the oxidase reagent was added to the suspension (8). Utilization of organic compounds as carbon and energy sources was tested in plates of basal medium BM (2) supplemented with 2 g of these compounds per liter.

DNA base composition. DNA was isolated by the method of Silhavy et al. (32). The G+C moles percent was calculated by high-pressure liquid chromatography (HPLC) determination of deoxynucleosides (11) using standard solutions of deoxynucleosides (0.1 mM) for calibration. The HPLC system was equipped with an SPD-M6A Shimadzu diode array detector, so that quantitation and identification by UV spectral analysis could be performed for every peak. Separation and determination of deoxynucleosides were carried out by using a Nova-Pak C_{18} column (4.6 mm by 25 cm) and an isocratic elution with 0.2 M ammonium dihydrogen phosphate adjusted to pH 4.5 containing 5% acetonitrile. DNA from phage lambda was digested and dephosphorylated under the same conditions to confirm the digestion and dephosphorylation, as well as the HPLC analysis.

Precursors for melanin synthesis. To check the utilization by strain MMB-1 of different monophenols as precursors for melanin synthesis, a medium called GEL, on which strain MMB-1 did not produce melanin, was chosen. This medium contained SST saline solution (20 g of NaCl, 7 g of $MgSO_4 \cdot 7H_2O$, 5.3 g of $MgCl_2 \cdot 6H_2O$, 0.7 g of KCl, 1.25 g of $CaCl_2$, and 6.1 g of Tris base per liter). The pH was adjusted to 7.4 with HCl (23) and supplemented with 7.5 mg of K_2HPO_4, 5 mg of $CuSO_4 \cdot 5H_2O$, 0.5 g of yeast extract, and 2 g of glucose per liter. In addition, the different monophenols (5 mM each) were added to the GEL to test their ability to be used as melanin precursors. The medium was inoculated with strain MMB-1 and incubated for 4 days at 25°C. Pigment formation was evidenced by the appearance of color and quantitated by measuring the absorbance at 400 nm in the supernatant of the culture media (30).

Enzymatic determinations. Bacterial cultures were centrifuged at 5,000 × *g* for 10 min to spin down the cellular pellet and to obtain clear supernatants. Cells were washed once with SST, resuspended in 1 ml of 0.1 M phosphate buffer (pH 5), and disrupted by use of a Braun Labsonic U sonicator (4-min treatments at a relative output power of 0.5 with 0.7 duty periods). The tubes were centrifuged at 13,000 × *g* for 15 min, and the supernatant was used as the crude cellular extract. Secreted proteins contained in the supernatant were obtained by precipitation with 80% saturation of ammonium sulfate.

The PPO activities of the cellular extract and the supernatant were spectrophotometrically determined at 37°C by recording the increase of absorbance at the appropriate wavelength depending on the substrate used. The most widely used were L-dopa, L-tyrosine (475 nm), 2,6-dimethoxyphenol (2,6-DMP) (470 nm), and syringaldazine (525 nm). The first two substrates can be considered tyrosinase substrates, and the last two can be considered laccase substrates. Our studies indicated that pH 5 is optimal for the tyrosine hydroxylase, L-dopa oxidase, and 2,6-DMP oxidase assays, but pH 6.5 was more convenient for the syringaldazine oxidase activity (see Results). The assay mixture (1 ml in all cases) contained 2 mM L-dopa, L-tyrosine, or 2,6-DMP in 0.1 M phosphate buffer, pH 5. For the tyrosine hydroxylase activity, the assay was also dependent on the addition of catalytic amounts of L-dopa and sodium dodecyl sulfate (SDS) (see Results). The reaction was initiated by the addition of cellular extract, usually 50 μl. For syringaldazine, due to its limited solubility in aqueous media and other characteristics (see Results), the reaction mixture consisted of 0.9 ml of 0.1 M phosphate buffer (pH 6.5) and 50 μl of the enzymatic sample. Then the assay was initiated by addition of 50 μl of 1 mM syringaldazine dissolved in absolute ethanol. In some assays, catalase (20 μg) was added to the assay solution to remove the possible effect of a bacterial peroxidase. The presence of catalase did not change the measurements by more than 10%. The hydroxylation of L-tyrosine and other monophenols was sometimes determined by recording serial UV-visible spectra at different times in the absence or the presence of 0.1% SDS to identify the product formed after the enzyme action. The reference cuvettes always had the same composition except for the substrate. One enzymatic unit was defined as the amount of enzyme that catalyzes the appearance of 1 μmol of product per min at 37°C. To estimate and compare the activities measured in this work, the following molecular extinction coefficients for the different oxidation products were used (per molar per centimeter): 3,700 for L-dopachrome (formed from L-tyrosine and L-dopa [22]), 65,000 for tetramethoxy-azo-bis-methylen quinone (from syringaldazine [11]), 5,570 for tetraguaiacol (from guaiacol [13]), and 14,800 for 3,3′,5,5′-tetramethoxy-diphenyl-quinone (from 2,6-DMP [33]).

Electrophoretic procedures. Analytical SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (20), using acrylamide concentrations of 11% for the separating gel and 3% for the stacking gel. The resolving buffer was Tris-HCl (pH 8.8) and the reservoir buffer was Tris-glycine (pH 8.3), both containing 0.1% SDS. Samples were mixed in a 2:1 (vol/vol) ratio with sample buffer (0.18 M Tris-HCl [pH 6.8], 15% glycerol, 0.075% bromophenol blue, 0.3% SDS). 2-Mercaptoethanol was omitted from the sample buffer for specific activity staining of gels. Electrophoresis was done at 20°C and at constant currents of 15 mA for 20 min and 30 mA for about 60 min.

Nondissociating PAGE was performed as described by Hames (12), using high-pH discontinuous buffers. The acrylamide concentrations were 11% for the separating gel and 3% for the stacking gel. Samples were mixed in a 2:1 (vol/vol) ratio with sample buffer (0.18 M Tris-HCl [pH 6.8], 15% glycerol, 0.075% bromophenol blue). Resolving and reservoir buffers were identical to the buffers used by Laemmli for dissociating electrophoresis but without SDS. Electrophoresis was also run under the same conditions as analytical SDS-PAGE.

Specific activity staining of gels. The slabs were equilibrated by immersion in 0.1 M phosphate buffer, pH 5 (tyrosine hydroxylase, L-dopa oxidase, or 2,6-DMP oxidase) or pH 6.5 (syringaldazine oxidase) for 5 min. Tyrosine and L-dopa oxidase specific activity stains were carried out by incubating the gels at 37°C in 1 mM L-tyrosine or L-dopa–2.5 mM MBTH (3-methyl-2-benzothizolinone hydrazone) in 50 mM phosphate buffer (pH 5.0) for 10 to 20 min (16). When appropriate, 0.02% SDS was also added to the staining mixture, 2,6-DMP oxidase was stained by incubation at 37°C in 2 mM 2,6-DMP in 0.1 M phosphate buffer (pH 5.0) for 10 to 20 min. Finally, syringaldazine oxidase was stained by incubation at 37°C in 0.1 mM syringaldazine in 0.1 M phosphate buffer (pH 6.5) for 10 min. After tyrosine hydroxylase, L-dopa oxidase, and 2,6-DMP oxidase staining, the gels could be dried because of the stability of the stain. Gels stained with

TABLE 1. Taxonomic characteristics of strain MMB-1 compared to the genus *Alteromonas* as described in *Bergey's Manual of Systematic Bacteriology* (3)[a]

Characteristic	MMB-1	*Alteromonas*
Morphology		
Straight rod	+	D[b]
Curved rod	−	D
Motility	+	+
Growth at:		
5°C	−	D
25°C	+	+
35°C	−	D
Flagellation		
Polar	+	+
Peritrichous	−	−
Anaerobic growth	−	−
Na^+ requirement	+	+
PHB accumulation	−	−
Denitrification	−	−
NO_3^- reduction to NO_2^-	+	D
Cytochrome *c* oxidase	−	+[c]
Luminescence	−	−[d]
Pigmentation	+	D
Lipase	+	D
Gelatinase	+	D
Agarase	−	D
Amylase	−	D
Utilization of:		
D-Glucose	+	+
D-Fructose	−	D
D-Mannose	+	D
D-Sorbitol	+	D
Maltose	−	D
Lactose	−	D
Citrate	+	D
β-Hydroxybutyrate	+	D
m-Hydroxybenzoate	−	D
Succinate	+	D
Malate	+	D
α-Ketoglutarate	−	D
Glycerol	+	D
Methanol	−	−
L-Tyrosine	−	D
% G+C (mean ± SD)	46.3 ± 0.9	38–50

[a] +, positive result; −, negative result.
[b] D, variable reactions depending on the species.
[c] *A. vaga* is negative.
[d] *A. hanedai* is positive.

syringaldazine could not be dried because of the instability of the product of the reaction.

Mutagenesis. Strain MMB-1 was mutagenized with nitrosoguanidine. Logarithmically growing cells in Marine Broth 2216 were inoculated in SST to obtain 0.2 U of initial absorbance at 600 nm. Nitrosoguanidine was added to a final concentration of 0.1 mg/ml. The suspension was incubated for different periods ranging from 5 to 15 min. At various intervals, the cells were diluted 1:10 in SST, centrifuged, washed, and resuspended in SST. Bacterial viability was assayed by cell counting on Marine Agar 2216. The remainder of the culture was inoculated in Marine Broth 2216. With nitrosoguanidine treatments of 5 to 10 min, a 10 to 5% viable fraction was obtained, so this mutagenesis time was chosen. The mutagenized cells grown in Marine Broth 2216 were plated on Marine Agar 2216 and screened for melanin synthesis.

RESULTS

Morphology and physiological characteristics. Strain MMB-1 appeared as a motile, gram-negative rod. Electron microscopy showed that it has a single polar, unsheathed flagellum. The most important selected physiological characteristics of strain MMB-1 analyzed in this study are summarized in Table 1.

DNA base composition. The G+C content of strain MMB-1 was calculated by HPLC determination of deoxynucleosides after DNA hydrolysis and dephosphorylation (17). The chromatograms obtained for DNA from strain MMB-1 showed, in addition to the expected four deoxynucleosides, two additional peaks which eluted at 6.77 and 12.78 min (Fig. 1). These peaks did not appear in the samples from phage lambda. Moreover, their UV absorption spectra had profiles very similar to the spectra of purine deoxynucleosides, as evidenced by the diode array detector, suggesting that they could be methylated derivatives. The possible existence of methylated deoxyguanosine in the digest from bacterial DNA seemed interesting. To further characterize these peaks, we also tried the commercially available O^6-methyl-2′-deoxyguanosine and 7-methyl-2′-deoxyguanosine-5′-diphosphate as standards. The latter was first dephosphorylated under the same conditions as the bacterial samples. Both standards gave peaks with retention times very close to that of the putative methylated deoxyguanosine that appeared in the bacterial DNA samples at 6.77 min. However, their adsorption spectra were not identical (data not shown). The unidentified compounds could be methylated purine deoxynucleosides, but complete identification was not possible at this stage.

The G+C content was calculated by using the areas of deoxycytidine and thymidine to avoid interferences with the methylated purine bases, obtaining a value of 46.3% ± 0.9%. This value is the mean ± standard deviation from duplicates of two independent experiments.

Melanin precursors and kinetics of pigment appearance. Strain MMB-1 synthesized a dark pigment when inoculated in complex medium (Marine Agar or Broth 2216). In Marine Broth 2216, the pigment appeared abruptly after 2 days of incubation, during the stationary phase of growth. In solid medium, the pigment was detected even later, in 4-day-old plates, both in the colonies and in the surrounding medium (data not shown).

In order to identify the precursors for melanin synthesis, a medium, GEL, in which strain MMB-1 did not synthesize melanin, was developed. Thus, different monophenols were added to GEL to test whether they could serve as melanin precursors (Table 2). It was observed that L-tyrosine was the only compound that acted as a precursor of melanin. Other monophenols with structures similar to that of L-tyrosine could not serve as pigment precursors. Furthermore, two closely related compounds, *p*-hydroxyphenylpyruvate and *p*-hydroxyphenylglycine,

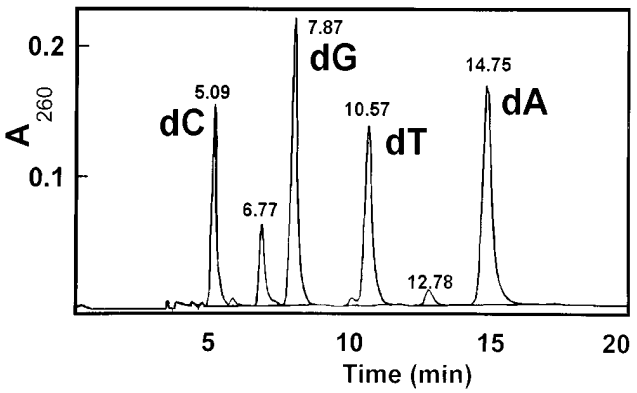

FIG. 1. HPLC chromatogram of DNA from strain MMB-1 which was digested with nuclease P1 and dephosphorylated. Deoxynucleosides dC, dG, dT, and dA were identified by the retention times and UV spectra.

TABLE 2. Utilization of different aromatic compounds by strain MMB-1 as putative melanin precursors

Compound added[a]	Growth[b]	Melanin formation (A_{400})[c]
None	+	− (0.15)
L-Tyrosine	+	+ (1.72)
L-Phenylalanine	+	− (0.15)
Gentisate	+	− (0.20)
m-Hydroxybenzoate	+	− (0.18)
p-Coumarate	+	− (0.21)
p-Hydroxymandelate	+	− (0.18)
p-Hydroxyphenylpropionate	+	− (0.13)
p-Hydroxyphenylpyruvate	−	− (0.17)
p-Hydroxyphenylglycine	−	− (0.11)

[a] The indicated phenols (5 mM) were added to GEL, and the cultures were incubated for 4 days at 25°C.
[b] +, growth; −, no growth.
[c] Melanin formation was determined by measuring the final absorbance of the culture supernatants at 400 nm. +, melanin formation as judged by visual criteria; −, no melanin formation.

not only failed as melanin precursors but also inhibited the growth of strain MMB-1.

Enzymatic activities. The ability of MMB-1 extracts to catalyze the oxidation of a number of substrates of tyrosinase and laccase was assayed in vitro in the cellular fraction and the extracellular culture media. Cellular extracts were able to oxidize a wide range of substrates, including overlapping substrates for tyrosinase and laccase, and also specific substrates, such as L-tyrosine and syringaldazine. Some PPO activity with L-dopa and 2,6-DMP as substrates was also detected in the supernatants of the cultures, but this activity was less than 5% in comparison to the activity found associated with the cells.

Therefore, we further explored the PPO activity detected in the cellular fraction. In a series of preliminary experiments, the optimal pHs for the oxidation of four PPO substrates, L-tyrosine, L-dopa, 2,6-DMP, and syringaldazine, were determined. The pH profile activity showed optimal oxidase activity around pH 5 for all activities but syringaldazine oxidase, which showed very low activity at pHs below 5.5 and optimal activity around pH 6.5 (Fig. 2). Therefore, pH 5 was selected for the first three substrates and pH 6.5 was selected for syringaldazine in further studies.

Under the initial standard conditions of the assay, 2 mM substrate in phosphate buffer (pH 5), the tyrosine hydroxylation activity by MMB-1 cellular extracts was poor and difficult to observe, mainly because of a lag period (Fig. 3a, curve 1). These results did not agree with the data obtained in vivo for the utilization of L-tyrosine as a melanin precursor. The lag period in cresolase activity is a well-known characteristic of many tyrosinases from different sources (15, 27). Furthermore, in some cases, the existence of latent tyrosinase that could be activated by different treatments, such as addition of detergents and proteolysis, has been described (25, 37). Thus, we assayed the tyrosine hydroxylase activity in the presence of 0.1% SDS added to the reaction media. Under these conditions, the catalysis by MMB-1 extracts was observed much more clearly than in the absence of the detergent (Fig. 3a, curve 2). Conversely, incubation of the bacterial extract with 0.1% SDS for 1 h produced a total inactivation of the tyrosine hydroxylase activity.

Moreover, the spectral changes of the accumulated product from L-tyrosine indicated the formation of L-dopachrome, as judged by the appearance of the two characteristic absorbance bands centered at 305 and 475 nm (Fig. 3b). This suggests the existence of an authentic tyrosinase in the bacterium, since this enzyme always shows inseparable cresolase and catecholase activities and therefore is able to catalyze directly the conversion of L-tyrosine into L-dopachrome.

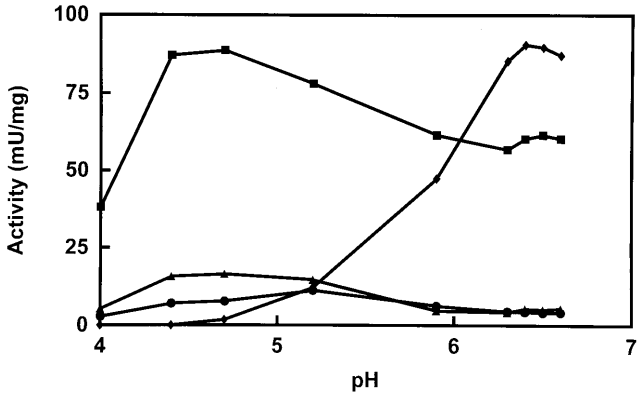

FIG. 2. Activity versus pH in the oxidation of different substrates by cellular extracts from strain MMB-1. pH 4 was obtained with 0.1 M sodium acetate buffer, and all higher pHs were prepared with 0.1 M sodium phosphate buffer. ●, L-tyrosine; ▲, L-dopa; ■, 2,6-DMP; and ◆, syringaldazine.

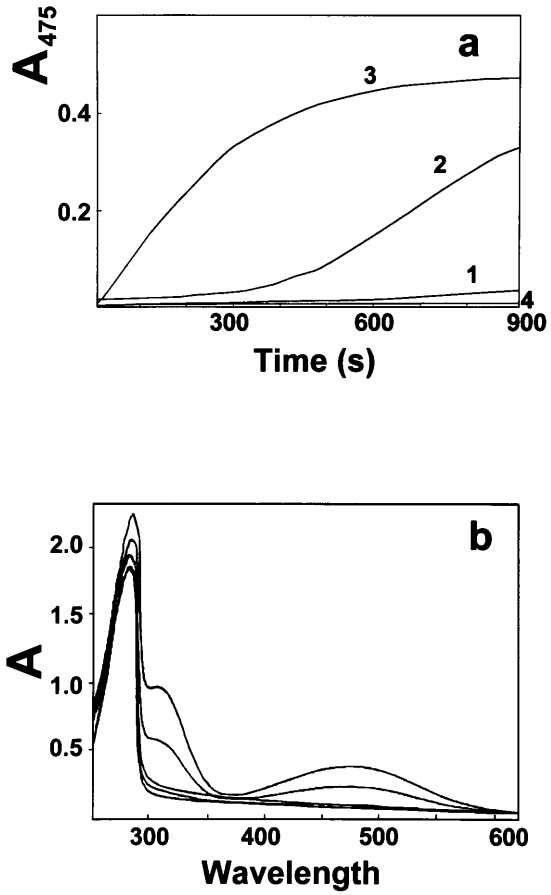

FIG. 3. L-Tyrosine oxidation by cellular extracts of strain MMB-1. (a) Curve 1, 2 mM L-Tyr; curve 2, same as curve 1 but in the presence of 0.1% SDS; curve 3, same as curve 1 but in the presence of 0.1% SDS plus 0.05 mM L-dopa as a cofactor; curve 4, 0.1% SDS plus 0.05 mM L-dopa without L-Tyr. (b) Serial UV-visible absorption spectra of sample 2. Spectra were recorded every 3 min (from 0 to 12 min).

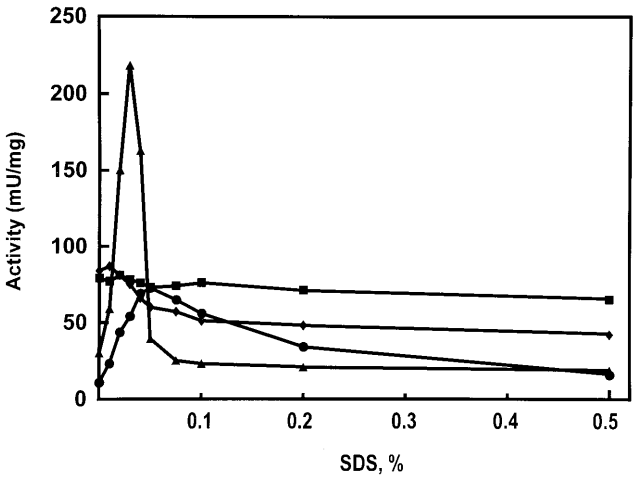

FIG. 4. Effect of SDS concentration on the oxidation of different substrates by cellular extracts of strain MMB-1. ●, L-tyrosine; ▲, L-dopa; ■, 2,6-DMP; and ◆, syringaldazine.

The lag period was shortened but not totally eliminated by the SDS. A further improvement of the assay for the tyrosine hydroxylase activity of tyrosinase was the addition of catalytic amounts of L-dopa as a cofactor for the reaction (15, 27). In the presence of 25 μM L-dopa, the lag period was eliminated (Fig. 3a, curve 3). The contribution of this small amount of L-dopa to L-dopachrome formation was negligible (Fig. 3a, curve 4). Thus, the optimal conditions for assaying the tyrosine hydroxylase activity of MMB-1 extracts required the addition of catalytic amounts of L-dopa and SDS to the assay mixture.

According to the results obtained in vivo for other monophenols that failed as melanin precursors (Table 2), in vitro experiments recording serial spectra confirmed that these phenolic compounds were not oxidized by the bacterial PPO even in the presence of 0.1% SDS (data not shown). This finding pointed out the stringent specificity of the bacterial cresolase activity for L-tyrosine as a unique substrate. Not only *p*-hydroxy acids lacking the amine group, such as *p*-hydroxyphenylacetic or *p*-hydroxyphenylpyruvic acid, but even other amine monophenols, such as *p*-hydroxyphenylglycine, were not recognized by the bacterial PPO.

The effect of SDS on the oxidation of the other substrates by MMB-1 extracts was also studied. Significantly, the addition of SDS below 0.05% also induced a remarkable activation of the L-dopa oxidase activity, but it tended to inhibit the oxidation of 2,6-DMP and syringaldazine (Fig. 4).

In order to determine the nature of the PPO detected in strain MMB-1, cellular extracts were compared to model tyrosinase and laccase in their ability to oxidize different phenolic substrates (Table 3). Mushroom tyrosinase and laccase from *Pyricularia oryzae* were used as standard PPOs. Confirming previous data, these model enzymes were not able to oxidize all substrates. L-Dopa and 2,6-DMP were substrates of both PPOs, but L-tyrosine was oxidized only by mushroom tyrosinase and syringaldazine was oxidized only by laccase. SDS caused inhibition of mushroom tyrosinase. The bacterial extracts from strain MMB-1 were able to oxidize the four substrates used, and their cresolase and catecholase activities were strongly activated by SDS. In addition, it is worth mentioning that for some substrates, such as 2,6-DMP, the specific activities of the MMB-1 extracts were even higher than those of the commercial enzymes, although the latter are at least partially purified. These data indicate the high activity of the bacterial PPO in comparison to those of eukaryotic oxidases.

Nitrosoguanidine mutagenesis. Several hundred colonies were screened after nitrosoguanidine mutagenesis. A great variability in the pigmentation of the colonies was observed, and those which were not pigmented were selected in a first screening. The absence of pigmentation in the selected strains was confirmed by several subcultures in the same medium. Finally, several mutants were chosen on the basis of their similar growth rates in Marine Broth 2216 and their capacity to grow in mineral media (data not shown). The representative mutant, strain NG56, was unable to synthesize melanin in any of the media tested, Marine Broth or Marine Agar 2216 or GEL plus 5 mM L-tyrosine. Study of the PPO activities showed that this mutant maintained the ability to oxidize all the substrates, and the specific activities were very similar to those of the original strain (Table 3). However, differing from the wild type, the cresolase and catecholase activities were not activated by the presence of SDS (Table 3). Thus, it is clear that the nitrosoguanidine treatment caused a loss of the detergent-dependent activation of the tyrosinase-preferred activities in the mutant strain. Similar results were obtained with other amelanogenic mutant strains.

PAGE. To analyze the possible heterogeneity of the PPO activities detected in strain MMB-1, cellular extracts of strains MMB-1 and NG56 were first subjected to PAGE under non-dissociating conditions. After electrophoresis, the gels were stained by immersion in solutions of the different PPO substrates, in the absence or presence of 0.02% SDS. In the wild-type and mutant extracts, a broad unresolved band was detected in the upper part of the gel. This band was stained with L-tyrosine, L-dopa, 2,6-DMP, and syringaldazine, showing that this fraction was able to oxidize all PPO substrates, regardless of their structure and the absence or presence of SDS during the staining (Fig. 5A and B). In addition to this multifunctional fraction, a lower band with high electrophoretic mobility was prominent in wild-type extracts. This band was stained only with L-dopa and L-tyrosine and was not stained with the laccase substrates, 2,6-DMP (Fig. 5B) and syringaldazine (not shown). In turn, this band was much more prominent in the MMB-1 extracts when the gels were stained in the presence of SDS (Fig. 5A), according to the detergent activation observed in the spectrophotometric determinations of the cresolase and catecholase activities in the wild-type extracts.

SDS-PAGE under dissociating conditions showed that the

TABLE 3. Specific activities of extracts from strain MMB-1, mutant NG56, and commercial solutions of the mushroom tyrosinase and laccase from *P. oryzae*[a]

Substrate	Sp act (mU/mg of protein)			
	MMB-1	NG56	Mushroom tyrosinase	Laccase from *P. oryzae*
L-Tyrosine	17 ± 6	8 ± 3	410 ± 24	ND[b]
L-Tyrosine + SDS	83 ± 10	10 ± 6	90 ± 22	ND
L-Dopa	51 ± 24	70 ± 20	2,968 ± 101	2.5 ± 0.5
L-Dopa + SDS	232 ± 80	73 ± 20	772 ± 64	1.5 ± 0.2
2,6-DMP	105 ± 37	90 ± 35	31 ± 3	36 ± 3
Guaiacol	9 ± 3	8 ± 2	5 ± 1	16 ± 2
Syringaldazine	91 ± 14	85 ± 20	ND	97 ± 9

[a] Commercial solutions were from Sigma Co. MMB-1 and NG56 cells were grown for 24 h in Marine Broth 2216. Values are means ± standard deviations (n = 5 for MMB-1 bacterial extracts and n = 3 for NG56 extracts and the commercial enzymes).

[b] ND, not detectable (<0.1 mU/mg).

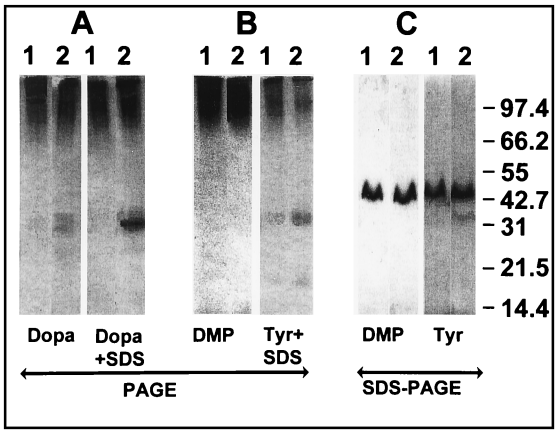

FIG. 5. Electrophoretic analysis of the PPO activity in the NG56 mutant strain (lanes 1) and the wild type, MMB-1 (lanes 2). (A) Gels were run under nondissociating conditions and stained with L-dopa in the absence or presence of 0.02% SDS as indicated below the lanes. (B) The same electrophoretic conditions as for panel A, but the stain was performed with 2,6-DMP and L-tyrosine plus SDS as indicated below the lanes. (C) Gels run under dissociating conditions and stained with 2,6-DMP and L-tyrosine as indicated. Calibration for apparent molecular masses was performed with standard proteins stained with Coomassie blue. Approximately 10 μg of protein was applied to each lane.

broad bands disappeared from the upper part of the gels and a new band was then detected in the extracts from both MMB-1 and the NG56 mutant. Moreover, this band showed all the PPO activities, regardless of the substrate used for gel staining (Fig. 5C). Protein staining and the use of standards indicated that this band has a molecular mass of around 46 kDa. In addition to this band, the wild-type MMB-1 showed a second, faint band of higher mobility (approximately 34 kDa) which could be stained only with tyrosinase substrates, L-tyrosine or L-dopa. This band was not stained with laccase substrates, and it was lacking in NG56 extracts. These properties were the same as those of the high-mobility band observed under nondissociating PAGE.

DISCUSSION

On the basis of morphology, polar flagellation, G+C content, aerobic metabolism, Na^+ requirement for growth, inability to accumulate PHB, and absence of denitrification, strain MMB-1 could be reasonably assigned to the genus *Alteromonas* as described in *Bergey's Manual of Systematic Bacteriology* (3). It is particularly interesting that strain MMB-1 appeared to be cytochrome *c* oxidase negative. As far as we know, only *Alteromonas vaga* shows this characteristic. However, *A. vaga* is not melanogenic, is gelatinase negative, and can use *m*-hydroxybenzoate as the sole carbon and energy source. All these features clearly differentiate this species from strain MMB-1.

A few *Alteromonas* species are able to synthesize melanins (3). However, strain MMB-1 differs from them in other characteristics in addition to the aforementioned absence of cytochrome *c* oxidase activity. No identified species is able to use sorbitol or malate as the sole carbon and energy source, unlike strain MMB-1. In addition, *Alteromonas hanedai* is luminescent (3), whereas strain MMB-1 is not. On the other hand, *Alteromonas luteoviolacea*, *Alteromonas nigrifaciens*, and *Alteromonas colwelliana*, lately reclassified as *S. colwelliana* (6), are amylase positive.

It is also worth noting that there is another marine melanogenic bacterium, ATCC 43961 (strain 2-40), whose taxonomic status is uncertain but which could likely be assigned to the genus *Alteromonas* (1). That strain clearly differs from strain MMB-1 in a number of characteristics. For example, strain 2-40 is amylase and agarase positive, so it causes depressions on the agar surface in Marine Agar 2216. Moreover, the melanin synthesized by strain 2-40 does not diffuse to the medium, and only the colonies are pigmented.

Concerning the DNA composition of MMB-1, the appearance of two peaks corresponding to purine deoxynucleosides after hydrolysis and dephosphorylation of the bacterial DNA is interesting. Our results do not allow for a clear identification of these peaks because of the lack of identical standards. However, the UV spectra of these peaks strongly suggest that the extra peak at 6.77 min may correspond to a deoxyguanosine methylated at a position other than 6 or 7, and the extra peak at 12.78 min may correspond to a methylated deoxyadenosine. As far as we know, there are no reports of the presence of methylated deoxyguanosines in other DNAs, so this feature is important. Further experiments with this bacterial DNA are needed to explore this very interesting possibility and its significance.

In conclusion, the physiological features of strain MMB-1 indicate that it does not fit into any previously described species in the genus *Alteromonas*. In order to establish its definitive taxonomic position in this heterogeneous genus, further studies, particularly rRNA sequencing, are necessary. Strain MMB-1 has been deposited in the Spanish Type Culture Collection as strain CECT 4803.

Concerning bacterial melanogenesis, our results show that strain MMB-1 synthesizes melanin by using L-tyrosine as a specific precursor and that it has a complex PPO system involved in pigment formation. This idea is supported by several lines of experimental evidence. First, in GEL medium, melanin did not appear unless L-tyrosine was added. This phenolic amino acid could not be substituted by other structurally related compounds, including its keto acid, *p*-hydroxyphenylpyruvate, and the amine analog *p*-hydroxyphenylglycine. Second, the oxidation of L-tyrosine catalyzed by cellular extracts of strain MMB-1 has been demonstrated in vitro, although the oxidation showed a lag period and was largely dependent on the presence of a small amount of L-dopa. Third, several nonpigmented mutants specifically affected in the SDS-dependent activation of the tyrosine hydroxylase activity of the wild-type strain have been isolated by nitrosoguanidine mutagenesis.

The PPO system detected in strain MMB-1 shows interesting properties. First, it is able to catalyze L-tyrosine hydroxylation. This cresolase activity is normally specific to tyrosinases, and it is lacking in all the laccases described to date. The oxidation of tyrosine directly yields L-dopachrome, as expected for an authentic tyrosinase, and this implies that L-dopa and L-dopaquinone are formed as intermediates. In fact, L-dopa is also a very good substrate for the PPO system of strain MMB-1, and this activity shows properties parallel to those of the tyrosine hydroxylase concerning optimum pH and activation by small amounts of SDS. However, the bacterial PPO also shows laccase-specific activity, since it is able to catalyze the oxidation of syringaldazine, a substrate specific for this kind of enzyme (7, 11, 35). Furthermore, cellular extracts of strain MMB-1 oxidize 2,6-DMP, a methoxy-substituted monophenol, more efficiently than model laccase from *P. oryzae* (Table 3). Thus, the PPO system from MMB-1 shares properties with tyrosinase and laccase.

The optimal pH values of the PPO activity for the different substrates lie well within the range determined for fungal PPOs. Furthermore, in agreement with findings for other PPOs obtained from fungi (*Agaricus bisporus*) or plants (broad

beans) (25), there is an acidic optimal pH and another, suboptimal region closer to a neutral pH. It is difficult to discuss the implications of this heterogeneity as well as the optimal pH for syringaldazine oxidation, since the purple dimeric product measured with this substrate is formed after the chemical coupling of two free radicals formed by the enzymatic action. Therefore, the pH dependence of the coupling reaction might affect detection of the optimal pH for the enzymatic action.

Most of the tyrosine hydroxylase activity of strain MMB-1 occurs in a latent form, and it is activated by addition of small amounts of SDS. Although the detergent requirement is not general for all tyrosinases (e.g., mushroom tyrosinase [Table 3]), it is typical and has been described for tyrosinases from plants (25) and animals (37). The maximal activation by SDS is achieved at a detergent concentration below the critical micelle concentration (i.e., less than 0.1% [25]), suggesting that its effect on PPO is limited to a conformational change due to binding of small amounts of SDS. The effect observed in vitro may reflect an in vivo activation of a latent PPO associated with a conformational change in the enzyme, although the mechanism and physiological significance of this process remain to be elucidated. The requirements of SDS and L-dopa for detection of a fully activated tyrosine hydroxylase activity strongly support our contention that strain MMB-1 expresses an authentic tyrosinase similar to the enzyme found in eukaryotic organisms (25, 27, 37). This hypothesis is reinforced by the fact that the L-dopa oxidase activity of PPO is also activated by SDS.

In contrast to the activation of the tyrosinase activities, cresolase and catecholase, the oxidation of 2,6-DMP and syringaldazine could not be activated by SDS. These results might suggest the existence of a single enzyme containing two different active sites, one tyrosinase-like site and another laccase-like site. As far as we know, a single PPO with those characteristics has never been described before. An alternative possibility is the coexistence of two different enzymatic systems in strain MMB-1, one latent tyrosinase, which could be activated by SDS and which would be able to catalyze the hydroxylation of L-tyrosine and the oxidation of L-dopa, and a second enzyme that would be a laccase-like PPO able to catalyze the oxidation of methoxyphenols, such as 2,6-DMP and syringaldazine. The coexistence of different PPOs has been described for some fungi, such as *Neurospora* (14), but never for bacteria. In fact, there is only one bacterium, isolated from soil, *Azospirillum lipoferum*, in which laccase activity has been revealed (11).

PAGE analysis of cellular extracts of the wild-type strain and the nonmelanogenic NG56 mutant seems to indicate the existence of two different PPOs. Specific staining of gels from the wild-type and mutant extracts showed the existence of a broad unresolved band stained with all substrates, supporting the observation that this mutant expressed all the PPO activities (Table 3). SDS-PAGE showed that the upper bands could be dissociated into a new band of 46 kDa detected also in both bacterial strains. This band still showed all the oxidase activities, supporting its relationship with the broad diffuse bands obtained under nondissociating conditions. The higher mobility of this multifunctional PPO under dissociating conditions could indicate the oligomeric nature of this enzyme. Thus, it can be concluded that SDS induced the dissociation of this enzyme but not its activation, since the enzyme levels in the mutant strain containing only this enzyme were not affected by the addition of the detergent.

The wild-type extracts contained another PPO band of higher mobility that was very faint or not detectable in the mutant NG56 cellular extracts. This was concomitant with the absence of SDS-activated tyrosine hydroxylase and L-dopa oxidase activities in the mutant extracts. That band showed tyrosine hydroxylase and L-dopa oxidase activities but no 2,6-DMP or syringaldazine oxidase activities. In addition, these enzymes were activated by the presence of 0.02% SDS during staining of gels run under nondissociating conditions. In contrast, under dissociating conditions, this PPO appears as a faint band. Thus, the tyrosine hydroxylase and L-dopa oxidase activities associated with this second enzyme would have been diminished in SDS-PAGE by the effect of the prolonged exposure to SDS during the electrophoresis. This agrees with the sensitivity of the cresolase and catecholase activities to incubation with the detergent. It can be concluded that SDS causes a double effect on this PPO, first a fast activation of the enzyme and then a slow inactivation.

In summary, PAGE analysis in the presence and absence of SDS unequivocally proves that MMB-1 has two different oxidases, one multifunctional PPO and another latent PPO showing tyrosinase characteristics that can be activated by SDS. The second PPO is lacking in the NG56 strain. Since NG56 was selected as nonpigmented mutants after nitrosoguanidine mutagenesis, it is obvious that the PPO involved in melanin synthesis is the SDS-activated PPO. The other oligomeric and pluripotent enzyme is unable to synthesize melanin under in vivo conditions, and its role remains to be elucidated.

The correlation between melanization and laccase activity in *A. lipoferum* was evidenced by the isolation of Tn*5* mutants affected in both characteristics (8). In contrast, the nonpigmented mutants of strain MMB-1 are specifically affected in the tyrosinase activities dependent on SDS activation. There is yet another report of a marine bacterium, strain 2-40, synthesizing a tyrosinase (18). This strain is also taxonomically related to the genus *Alteromonas*, as discussed above. Unlike the situation found for strain MMB-1, the tyrosinase present in strain 2-40 is not latent, and its tyrosine hydroxylase activity can be observed in the absence of detergent. All together, the data for *A. lipoferum* strains, 2-40, and MMB-1 point out the existence of different mechanisms of bacterial pigment formation with respect to the characteristics of the enzymes involved and the nature of pigment precursors.

In conclusion, the results presented here show that strain MMB-1 is a previously unidentified melanogenic bacterium. This strain has two PPOs. One is a tyrosinase-like latent enzyme activated by SDS in vitro and involved in melanogenesis from L-tyrosine. The other PPO shows unique properties that have never been described before for any other organism, since it shows cresolase, catecholase, and laccase activities. According to this finding, the separation between tyrosinase and laccase in the bacterial kingdom is not as clear as proposed for the PPOs obtained from eukaryotic cells, and the classification of PPOs as tyrosinases and laccases should be reconsidered.

The purification and characterization of the multifunctional PPO of strain MMB-1 may have important applications in bioremediation, lignin degradation, food processing, and cosmetic industries (4, 9, 26).

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