Melanin Produced by the Fast-Growing Marine Bacterium Vibrio natriegens through Heterologous Biosynthesis: Characterization and Application

Zheng Wang,a Tanya Tschirhart,a Zachary Schultzhaus,b Erin E. Kelly,a Amy Chen,a Eunkeu Oh,c Okhil Nag,a Evan R. Glaser,d Eunkyoung Kim,e Pamela F. Lloyd,f Paul T. Charles,a Wei Yao Li,g Dagmar Leary,a Jaimee Compton,a Daniel A. Phillips,b Ali Dhinojwala,g Gregory F. Payne,e and Gary J. Vora

Center for Biomolecular Science and Engineering, Naval Research Laboratory, Washington, DC, USA
National Research Council Postdoctoral Research Associate, Naval Research Laboratory, Washington, DC, USA
Optical Sciences Division, Naval Research Laboratory, Washington, DC, USA
Division of Electronics Science and Technology, Naval Research Laboratory, Washington, DC, USA
Institute for Bioscience and Biotechnology Research, University of Maryland, College Park, Maryland, USA
UES, Inc., Wright-Patterson Air Force Base, Ohio, USA
Department of Polymer Science, The University of Akron, Akron, Ohio, USA

ABSTRACT Melanin is a pigment produced by organisms throughout all domains of life. Due to its unique physicochemical properties, biocompatibility, and biostability, there has been an increasing interest in the use of melanin for broad applications. In the vast majority of studies, melanin has been either chemically synthesized or isolated from animals, which has restricted its use to small-scale applications. Using bacteria as biocatalysts is a promising and economical alternative for the large-scale production of biomaterials. In this study, we engineered the marine bacterium Vibrio natriegens, one of the fastest-growing organisms, to synthesize melanin by expressing a heterologous tyrosinase gene and demonstrated that melanin production was much faster than in previously reported heterologous systems. The melanin of V. natriegens was characterized as a polymer derived from dihydroxyindole-2-carboxylic acid (DHICA) and, similarly to synthetic melanin, exhibited several characteristic and useful features. Electron microscopy analysis demonstrated that melanin produced from V. natriegens formed nanoparticles that were assembled as “melanin ghost” structures, and the photoprotective properties of these particles were validated by their protection of cells from UV irradiation. Using a novel electrochemical reverse engineering method, we observed that melanization conferred redox activity to V. natriegens. Moreover, melanized bacteria were able to quickly adsorb the organic compound trinitrotoluene (TNT). Overall, the genetic tractability, rapid division time, and ease of culture provide a set of attractive properties that compare favorably to current E. coli production strains and warrant the further development of this chassis as a microbial factory for natural product biosynthesis.

IMPORTANCE Melanins are macromolecules that are ubiquitous in nature and impart a large variety of biological functions, including structure, coloration, radiation resistance, free radical scavenging, and thermoregulation. Currently, in the majority of investigations, melanins are either chemically synthesized or extracted from animals, which presents significant challenges for large-scale production. Bacteria have been used as biocatalysts to synthesize a variety of biomaterials due to their fast growth and amenability to genetic engineering using synthetic biology tools. In this study, we engineered the extremely fast-growing bacterium V. natriegens to synthesize melanin nanoparticles by expressing a heterologous tyrosinase gene with inducible promoters. Characterization of the melanin produced from V. natriegens-
produced tyrosinase revealed that it exhibited physical and chemical properties similar to those of natural and chemically synthesized melamins, including nanoparticle structure, protection against UV damage, and adsorption of toxic compounds. We anticipate that producing and controlling melanin structures at the nanoscale in this bacterial system with synthetic biology tools will enable the design and rapid production of novel biomaterials for multiple applications.

**KEYWORDS** Vibrio natriegens, biomanufacturing, fast growing, melanin, melanin biosynthesis, nanoparticle, synthetic biology

Melamins are macromolecules formed by oxidative polymerization of phenolic and/or indolic compounds. These black or brown pigments are hydrophobic, negatively charged, and ubiquitous in nature and impart a large variety of biological functions to organisms, including structure, coloration, free radical scavenging, radiation resistance, and thermoregulation (1). Inspired by the physicochemical, optoelectronic, self-assembling, and adhesive properties of natural melanin, a number of research groups have synthesized melanin nanoparticles for a broad range of applications, including protective coatings, functional films, environmental sensors, and energy storage devices (2, 3) Currently, however, commercially available melamins are either chemically synthesized (4) or extracted from sepia (5), and both approaches contain significant challenges for the generation of yields amenable to large-scale applications. Since microorganisms are easily cultivated and economically sustainable, they show great potential to produce advanced biomaterials. Many bacteria isolated from nature, including species of Bacillus, Aeromonas, Rhizobium, and Streptomyces, were reported to produce melanin via tyrosinase (monophenol monoxygenase EC 1.14.18.1), a copper-containing enzyme (6–11). The enzyme catalyzes the oxidation of \(\text{L-tyrosine} \rightarrow \text{o-dihydroxyphenylalanine (DOPA)} \) and dopaquinone, which undergoes cyclization to 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and further polymerizes spontaneously into melanin (see Fig. S1 in the supplemental material) (12). With recombinant DNA technology, the tyrosinase gene has previously been cloned and heterologously expressed in *Escherichia coli*, and the resulting melanin was tested in a variety of applications (13, 14).

The broad application of biomaterials like melanin on a large scale requires high productivity. However, yields of melanin production from various bacteria are determined by the quantities of substrates (\(\text{L-tyrosine} \) or \(\text{L-DOPA} \)) that are fed into the growth cultures and routed toward processing by tyrosinase, so improving melanin production efficiency has been achieved by natural selection of active enzymes or growth optimization (15, 16). Two of the most critical physiological features that impact a microbial production system are the growth rate and biomass-specific substrate uptake rate (17). Therefore, faster-growing organisms provide clear advantages over slower-growing organisms in industrial production. *E. coli* is often the gold-standard organism for genetic and metabolic engineering efforts, but it exhibits a \(\sim 40\)-min doubling time in glucose minimal medium (17). Alternatively, the Gram-negative marine bacterium *Vibrio natriegens* is recognized as one of the fastest-growing organisms currently known, with a reported doubling time of less than 10 min in rich medium and of less than 25 min in glucose minimal medium (18–21). With nutritional versatility, a high growth rate, and lack of pathogenicity, *V. natriegens* has become an attractive alternative to *E. coli* for biotechnological applications (22, 23). Additionally, a number of genetic tools have been developed to engineer *V. natriegens* (20, 21, 24) which make it an attractive chassis for synthetic biology, metabolic engineering, and biomaterial production.

In this study, we engineered *V. natriegens* to synthesize melanin by expressing a tyrosinase gene from Bacillus megaterium under the control of inducible promoters and demonstrated that *V. natriegens* was able to produce melanin faster than were previously reported heterologous systems. We found that the melanin produced from *V. natriegens*-produced tyrosinase could be found in cell-free supernatants as nanopar-
articles as well as associated with the cell wall, and it exhibited physical and chemical characteristics similar to those of natural and chemically synthesized melamins. This study demonstrated that *V. natriegens* could be used as a biocatalyst for fast production of a biopolymer.

**RESULTS**

**Expression of the tyrosinase gene and melanin production in *V. natriegens***. The tyrosinase gene *tyr1* from *B. megaterium* (25) was synthesized and placed under the control of the Ptac promoter and the *lacIq* repressor in vector pJV298 (26) using the method described by Tschirhart et al. (20) (Fig. S2). This plasmid was electroporated into *V. natriegens*, and after overnight incubation at 30°C, transformants (pJV-Tyr1) with black and diffusible pigments were observed on M9 agar plates supplemented with isopropyl-β-D-1-thiogalactopyranoside (IPTG) and L-tyrosine (data not shown). To verify the pigment identity, both pigmented and nonpigmented cells were incubated with anti-melanin antibodies. Fluorescence microscopy showed that immunofluorescence signals were localized on the black cells only (Fig. S3). This result confirmed that the pigments formed were melamins that were associated with the *V. natriegens* cells.

To measure tyrosinase expression and the efficacy of this construct, the recombinant strain was grown for 2 h to log phase and induced by IPTG in LBv2 liquid medium for 3 h. Cells were then collected for proteomic analysis. As assessed by shotgun proteomics, tyrosinase expression increased approximately 100-fold by induction in four biological replicates (Table 1), indicating that the Ptac promoter was efficiently regulated in *V. natriegens*. Induced cells were then resuspended in LBv2 and M9 liquid media with added L-tyrosine (0.4mg/ml) and CuSO4 respectively. Within 15 minutes, a black pigment was observed in the M9 culture (Fig. 1A), and melanin yield reached the maximal level (~0.45mg/ml) within 2 h, with a rate of approximately 0.32 mg/ml per hour (Fig. 1B), which was equivalent to 420 mg melanin (mel)/g cell dry weight (CDW)/h and significantly faster than any other melanin-producing microorganism (Table 2).

Theoretically, 1 g of L-tyrosine is expected to make 1.15 g of melanin by incorporating one atom of oxygen to the L-tyrosine molecule resulting from the tyrosine hydroxylase activity of tyrosinase (14). The melanin yield from this study suggested that L-tyrosine was almost completely converted into melanin by the recombinant bacteria. However, it was noted that the melanin production rate was significantly lower in the LBv2 culture, with a rate of ~0.05 mg/ml per hour (equivalent to 66 mg mel/gCDW/h), and melanin was saturated after 8 h with less yield than in M9 culture (Fig. 2B). We also found that some tyrosinase was released from the cells, as some tyrosinase activity could be detected in the supernatant of induced cells in M9 culture that had been filtered prior to the addition of L-tyrosine (Fig. 1A), indicating that shifting bacterial cells into the minimal medium resulted in the release of the expressed tyrosinase into the medium, perhaps from increased permeability or cell lysis, so as to accelerate accessing the substrates. To determine the effect of L-tyrosine quantity on melanin yields, M9 cultures containing IPTG-induced cells were supplemented with a range of L-tyrosine concentrations, from 0.1 mg/ml to 1 mg/ml. Figure 1C shows that the melanin production rates were very similar and that concentrations of synthesized melanin were correlated with concentrations of L-tyrosine under 0.6 mg/ml. Starting with an L-tyrosine concentration over 0.6 mg/ml resulted in concentrations of melanin that were above the detection limit of the assay due to product aggregation, which was reflected by fluctuation in the measurements at the later stage.

### TABLE 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Avg area ratio induced/uninduced for culture:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VN1</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>350</td>
</tr>
<tr>
<td>DnaK</td>
<td>2</td>
</tr>
</tbody>
</table>

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Expression of tyrosinase gene and melanin production in *V. natriegens* through IPTG induction. (A) Left, melanin production in M9 medium supplemented with 40 μg/ml CuSO4 and 0.4 mg/ml L-tyrosine after tyrosinase was induced in rich medium; right, melanin production from the filtered supernatant supplemented with CuSO4 and L-tyrosine after the induced cells were incubated in M9 medium for 15 min. (B) Kinetics of melanin production in M9 (●) and LBv2 (●) media supplemented with 40 μg/ml CuSO4 and L-tyrosine after tyrosinase was induced in rich medium. (C) Effect of L-tyrosine concentration (in milligrams per milliliter) on melanin production in M9 medium. Graphs in panels B and C represent the averages of the results from five independent experiments. (D) Syntheses of melanin variants with tripeptide precursors. IPTG-induced *V. natriegens*/pJV-Tyr1 cells were transferred into M9 medium supplemented with CuSO4 and 5 mM tripeptides and incubated at 37°C for 1 h.
Tyrosinase has been demonstrated to catalyze a variety of substrates, including short peptides containing L-tyrosine, for the synthesis of melanin-like polymeric pigments with diverse physicochemical properties in vitro (27). To determine the ability of *V. natriegens*-produced tyrosinase to catalyze the formation of melanin from multiple substrates, we added four tripeptides (DFY, FDF, DDF, and YFD) to tyrosinase-producing cultures of *V. natriegens* at 5 mM. Orange pigments with a variety of color intensities were observed in cell cultures after 2 h (Fig. 1D). In agreement with the in vitro experiment (28), tripeptides that contain paired aromatics (DFY, DDF, and YFD) gave rise to a relatively darker orange color than that of the tripeptide that has the pair separated by a charged aspartic acid. This result indicates that the paired aromatics in tripeptides not only tend to aggregate during the self-assembly process but also enhance the polymerization of oxidized peptides. It also suggests that tyrosinase-expressing *V. natriegens* can be used to produce functionalized melanin-like biopolymers with tunable properties by using various L-tyrosine-containing short peptides as the substrates.

**Induction of melanin production with optogenetics.** In an industrial setting, it may be desirable to have temporal control over the production of certain molecules in a chassis organism. Nonchemical induction using optogenetics offers many exciting opportunities and applications for controlling cell responses in programmable ways and is becoming a valuable part of synthetic biology circuits. Therefore, we also tested

![TABLE 2 Comparison of melanin production levels in different microbial hosts](http://aem.asm.org/)

<table>
<thead>
<tr>
<th>Host</th>
<th>Gene</th>
<th>Tm (°C)</th>
<th>Production time (h)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus weihenstephanensis</em></td>
<td>Laccase&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30</td>
<td>120</td>
<td>50</td>
</tr>
<tr>
<td><em>Streptomyces kathirae</em></td>
<td>Tyrosinase&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28</td>
<td>128</td>
<td>15</td>
</tr>
<tr>
<td><em>Streptomyces glaucescens</em></td>
<td>Tyrosinase&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30, 37</td>
<td>&gt;48</td>
<td>9</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>MelA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>30</td>
<td>30</td>
<td>14</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Tyr&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30</td>
<td>33</td>
<td>13</td>
</tr>
<tr>
<td><em>V. natriegens</em></td>
<td>Tyr&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30, 37</td>
<td>&lt;10</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup>Tm, growth temperature.  
<sup>b</sup>Production time is counted as from the start of bacterial growth to the beginning of melanin saturation period.  
<sup>c</sup>Endogenous gene.  
<sup>d</sup>Heterologously expressed gene.

![FIG 2 Induction of tyrosinase gene expression and melanin production in *V. natriegens* in an optogenetic system](http://aem.asm.org/)

A. pDawn-Tyrosinase

![Blue light](http://aem.asm.org/)

B. Light off on

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Melanin Production in *Vibrio natriegens*  
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aem.asm.org
induction of tyrosinase in V. natriegens using the light-driven pDawn system (Fig. 2A) (29). In the absence of blue light, the histidine kinase YF1, which contains a photosensory domain, phosphorylates its cognate response regulator FixJ and drives robust gene expression of θ repressor cI from the FixK2 promoter. The cI repressor prevents tyrosinase expression. Upon light exposure, a significant reduction in kinase activity of YF1 and, consequently, cI expression results in derepression of the pR promoter and tyrosinase expression, which then catalyzes melanin production in the presence of L-tyrosine. To control light input, we built the 48-well-plate-fitted light plate array (LPA) designed by the Tabor lab (30) to test our constructs. As can be seen in Fig. 2B, with the tested parameters, we saw melanin production in V. natriegens upon induction with light and no melanin where no light was applied. This proof-of-concept experiment demonstrated induction of a bioproduct through optogenetics in V. natriegens.

Bacterial melanin nanoparticles and “melanin ghosts.” Due to their high biocompatibility, melanin nanoparticles have been investigated in many biomedical and material applications and been made from chemical or enzymatic oxidation of the precursors L-DOPA or dopamine (27). In order to determine whether the recombinant bacterium was able to make melanin nanoparticles, the black supernatant separated from the V. natriegens culture 30 min after melanization and was subjected to transmission electron microscopy (TEM) and dynamic light scattering (DLS) analyses. TEM revealed uniform black particles of less than 20 nm, and DLS measurements showed a dominant cluster with particle sizes around 20 nm as well (Fig. 3A). However, these particles were not observed in the supernatant from the nonmelanized cell cultures. In the nonmelanized sample, two large peaks with small percentages of particle numbers were observed, and they might be attributed to by-products of cell culture extracts. Moreover, the sizes of melanin nanoparticles in the supernatant 12 h after melanization were around 70 nm (Fig. 3A), suggesting that longer incubation times increased the size of the nanoparticles. The addition of an extra 100 mM NaCl to the M9 medium resulted in larger particles (larger than 100 nm; Fig. 3A) and spontaneous precipitation of melanin aggregates, indicating that the sizes of melanin particles were also affected by salt concentration.

Melanin synthesized from other microbes such as fungi is typically located within the cell wall and plays a significant role in maintaining cell wall integrity (31). Melanin structures associated with the fungal cell walls, so-called “melanin ghosts,” are generated by exposure of melanized cells to 4 M guanidinium isothiocyanate followed by 6 M HCl at 100°C. This protocol was applied to treat melanized and nonmelanized V. natriegens cells. Under the same chemical treatment conditions, the nonmelanized bacterial cells were completely solubilized, but the melanized cells remained as black particulate materials. Scanning electron microscopy (SEM) images showed that these black particles exhibited morphologies similar to those of bacterial cells (Fig. 3B). It was also noted that there were nanosized granules on the surface, which might be melanin aggregates resulting from acid precipitation. In contrast to fungal melanin ghosts that had melanin walls without internal structures, TEM revealed that the bacterial black particles were filled with irregular electron-dense structures with no defined organelles or cell wall layers (Fig. 3B). This result showed that melanin ghost-like structures could be generated from bacteria as well even after other cellular components were depleted by acid hydrolysis.

Chemical characterization of melanin produced from V. natriegens. To verify the chemical composition of melanin produced from V. natriegens, black powder was extracted from the supernatant of melanized bacteria and analyzed with Fourier transform infrared (FTIR) spectroscopy. A comparison of the bacterium-producing melanin and synthetic melanin revealed significant similarities between IR spectra (Fig. 4A) representing equivalent functional groups, such as the hydrogen bond of an -OH group, an alkyl -CH2 group, and an aromatic ring including a C=H group. Notably, a peak at 1,722 cm⁻¹ suggested a C=O carbonyl group vibration, indicating the carboxylic acid from dihydroxyindole-2-carboxylic acid (DHICA).
Stable free radicals are characteristic of melanin and have a unique electron paramagnetic resonance (EPR) signal. To examine this signal in bacterial synthesized melanin, equal amounts of extracted melanin powders, melanized bacterial cells, bacterial melanin ghosts, and nonmelanized cells were examined using EPR (Fig. 4B).

**FIG 3** Extracellular melanin particles and bacterial melanin ghosts. (A) a, TEM image of melanin nanoparticles in the supernatant of *V. natriegens* cell culture after L-tyrosine addition in the bacterial culture at 30 min. b, hydrodynamic sizes of melanin nanoparticles measured by DLS. Melanin nanoparticles were formed after the addition of L-tyrosine in the presence of 250 mM NaCl. The measured average sizes were 16 ± 0.6 nm with L-tyrosine addition (similar to melanin nanoparticle measurement by TEM) and 97 ± 39 nm for the control without L-tyrosine addition that were attributed to the by-products of cell culture extract. c, melanin nanoparticles formed after L-tyrosine addition at 12 h with different concentrations of salt. The peaks showed at 35 ± 3.4 nm (250 mM NaCl, blue), 73 ± 5.3 nm (350 mM NaCl, orange), and 86 ± 9.6 nm (550 mM NaCl, gray). (B) SEM and TEM images of melanin ghosts and melanized bacterial cells.

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FIG 4 Characterization of melanin produced from V. natriegens. (A) FTIR spectra of bacterial (Bac) melanin and synthetic (Syn) melanin. Peaks in common and their assignments are 1,625 cm$^{-1}$, C=C vibration in (Continued on next page)
The first three samples exhibited distinct, stable free radical signals with Zeeman splitting g-values of 2.004 that are highly similar to synthetic eumelanin, fungal 1,8-dihydroxynaphthalene (DHN)-melanin, and bacterial pyomelanin (22). It was noted that melanin ghosts had the relatively highest signal intensity, and extracted melanin powders had the weakest signals. As expected, nonmelanized cells did not show an EPR signal. Illumination of melanin ghosts and melanized bacteria with UV-visible light from a xenon (Xe) lamp for 30 min increased the EPR signals (Fig. 4C).

Redox activity of melanized bacteria. We employed an electrochemical reverse engineering method (40) to characterize the redox activity of melanized V. natriegens cells. Figure 5A shows the schematic of the electrochemical reverse engineering method. Here, the melanized V. natriegens cells were immobilized at an electrode surface by codepositing it within a permeable alginate hydrogel film. The film-entrapped cells were probed for redox activity by immersing the film-coated electrode into a solution containing two diffusible mediators that can freely diffuse through the matrix and access the electrode. When the underlying electrode is cycled to oxidative (more positive) voltages, one mediator (50 μM ferrocene dimethanol [Fc]) can undergo oxidative redox cycling, in which Fc donates an electron to the electrode, the oxidized form (Fc+) diffuses into the film and accepts an electron from melanin, and this rereduced Fc mediator can then diffuse back to the electrode where it can be reoxidized by donating the electron to the electrode. This oxidative redox cycling serves to extract electrons from the melanin. The other mediator (50 μM Ru(NH₃)₆Cl₃ [Ru³⁺]) can undergo reductive redox cycling when the electrode potential is cycled to a reducing (more negative) voltage, in which Ru³⁺ accepts an electron from the electrode; the reduced form, Ru²⁺, diffuses into the film and donates its electron to melanin; and the reoxidized Ru³⁺ form diffuses back to the electrode where it can be rereduced. This reductive redox cycling serves to transfer electrons from the electrode to the melanin.

To control these redox cycling processes, an oscillating input voltage is imposed to the electrode (Fig. 5B). As the redox cycling processes occurs, the output currents associated with mediator oxidation/reduction reactions are measured. Two negative controls including the alginate-only film and the alginate film with nonmelanized V. natriegens showed small output peak currents. However, the melanized V. natriegens-alginate film-coated electrode showed large peak currents for both Ru³⁺ reduction and Fc oxidation (Fig. 5B). The high amplification of the redox currents provides evidence that the melanin produced by V. natriegens confers redox activity. To test the reversibility of this redox activity, the imposed input potential was repeatedly cycled over 150 min, as shown in Fig. 5C. The output current curve shows that when the melanized V. natriegens cells were embedded in the film, the mediator currents were amplified during both oxidation and reduction. Importantly, this amplification remained nearly steady over 2 h. This result indicates that the melanin produced by V. natriegens can be reversibly oxidized and reduced, and thus, the melanin can be repeatedly switched between redox states.

UV protection. Melanin has been demonstrated to have a role in protecting against oxidative stresses such as UV irradiation (33). When washed melanized and non-melanized V. natriegens cells suspended in phosphate-buffered saline (PBS) were irradiated with UVC, both types of cells were killed completely, indicating that the melanin in the cell walls was not sufficient to protect the cells. However, we did observe the protective property of melanin when melanized cell culture in the growth medium supplemented with l-tyrosine was exposed to UVC. UV at 450 mJ/cm² completely killed
bacterial cells in the nonmelanized culture (without adding L-tyrosine), but the cells in the melanized culture survived (Fig. 6A). Furthermore, supernatants filtered from the nonmelanized and melanized cell cultures were added into HeLa cell cultures, which were then irradiated with 4 mJ/cm² UVC. Figure 6B shows that more than 90% of HeLa cells mixed with the melanized supernatant were still alive after UVC irradiation, but

**FIG 5** Redox activity of melanized *V. natriegens* (*V. nat*) revealed by reverse electrical engineering method. (A) Schematic shows that melanin can donate/accept electrons to/from mediators by oxidative/reductive redox cycling process (details are described in the supplemental material). (B) The imposed input potential (i.e., voltage) and observed output current response associated with Ru³⁺ and Fc mediators. (C) Long-term cyclic experiments test for the reversibility of redox-activity; steady amplifications are signatures of reversible and repeated oxidation and reduction of melanin. E(V), potential in volts.

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less than 20% of cells mixed with the nonmelanized supernatant survived under the same conditions. Therefore, the extracellular melanin nanoparticles produced here have the ability to protect mammalian cells from UVC irradiation.

**TNT adsorption.** Melanin also exhibits promiscuous binding capabilities as a result of its complex polymeric structure. This situates melanin-producing bacteria as candidates for bioremediation applications. We therefore explored the ability of melanin-
producing *V. natriegens* to bind and sequester the common environmental contaminant 2,4,6-trinitrotoluene (TNT), an explosive material. We added melanin-producing *V. natriegens* to a solution of TNT with a final concentration of 125 ppb in PBS and observed that melanized cells could bind and remove most of the compound from solution. This process was time dependent, with only ~25% of TNT removed after a 5-min incubation but nearly 100% removed after a 30-min incubation (Fig. 7A). Additionally, the binding of TNT was pH dependent, with the efficiency of TNT removal from solution going from nearly 100% at pH 6, to 80% at pH 5, and to only 10% at pH 4 and pH 3 (Fig. 7B).

**DISCUSSION**

Melanin production by tyrosinase has been described in several bacterial species (9, 25, 34–37). Due to their unique physiochemical properties and potential industrial applications, it is desirable to produce melanins at large scale and low cost. Heterologous expression of tyrosinase genes in *E. coli* has been reported to produce melanins when growth media are supplemented with L-tyrosine, but melanin synthesis was slow, and melanins were fully produced only after at least 30 h (Table 2). We previously demonstrated that genetic modules adopted from *E. coli* functioned similarly in *V. natriegens* (20). The successful regulation of tyrosinase gene expression and melanin production through engineering inducible promoters in *V. natriegens* supports that this fast-growing marine bacterium represents a new synthetic biology chassis able to host a broad range of the genetic circuits and modulate diverse biomaterial manufactures. The proteomics analysis also validated that the expression of tyrosinase was steadily regulated by the Ptac promoter in *V. natriegens*. With a growth rate that is two times...
higher than that of *E. coli*, *V. natriegens* has a clear advantage as a biomanufacturing host by significantly reducing fermentation time, which will result in an economical benefit for large-scale melanin production. It was noted that, with the substrate concentrations below 0.6 mg/ml, melanin production accumulated to a maximal level and then became steady probably due to complete consumption of L-tyrosine. However, adding the substrate L-tyrosine at a concentration of or higher than 0.6 mg/ml resulted in melanin aggregation in the media due to solubility limitations at higher concentrations of these final products (Fig. 1C). We also demonstrated induction of this biopigment through optogenetics in *V. natriegens*. This exciting avenue not only illustrates that this fast-growing bacterium is an excellent chassis to host synthetic biology circuits but also revealed its great potential to make novel sensing and protective biomaterials in response to easily manipulated external signals. Since optogenetic systems that respond to UV light are available (30), they could be used to trigger melanin production to protect the cells from further UV damage or create patterns based on UV exposure.

Previously, *V. natriegens* was demonstrated to speed up selenium nanoparticle production by its own reducing capability (38). Our study suggested that *V. natriegens* could be used as a fast biocatalyst for biopolymer production. In this study, the tyrosinase was not tagged with the adhesin involved in diffuse adherence (AIDA) autotransporter vehicle for surface expression like in the previous investigation in *E. coli* (13), but melanins were not only found to be distributed in the cytoplasm and the cell surface by electronic microscopy but also largely produced in the cell cultures as nanoparticles (Fig. 3). Moreover, *V. natriegens* might be more susceptible to changes in pH and osmolarity than is *E. coli*, which can result in increased cell permeability or cell lysis, especially where *V. natriegens* is transferred from rich medium to minimal medium (Fig. 1A). This susceptibility resulted in the release of tyrosinase into growth media that accelerated melanin production and could potentially result in lower downstream cost due to the reduced energy or time required to break open cells for protein harvest.

The formation of melanin nanoparticles has been well described in chemical reactions (39) and usually takes more than 12 h. In this study, we demonstrated that bacteria were able to produce melanin nanoparticles (MNPs) in as little as 30 min in cell culture (Fig. 4A). Correlations of particle sizes to incubation time and salt concentrations in the media (Fig. 3A) indicated that the sizes of bacteria producing MNPs could be tuned by controlling growth conditions. Melanin ghosts have been hallmarks for the study of melanin in fungi and are very stable after harsh hydrolysis treatment. Interestingly, we demonstrated that melanin ghosts could be formed in melanized bacteria as well, but melanin not only aggregated on the cell surface but also in the internal structure of ghosts (Fig. 4B), which was different from the fungal melanin ghosts that melanin assembled in the fungal cell walls. Heterogeneity due to cellular components and spherical morphologies, therefore, appears to provide melanin ghosts with novel properties not exhibited by pure melanins.

Both EPR and electrochemical analyses demonstrated that the melanized *V. natriegens* cells possess stable free radical scavenger and active redox properties. These properties will render additional and broader biological functions to this fast-growing bacterium. Along with mechanical resistance, the melanized bacteria may be a synthetic biology chassis well suited for functioning in extreme environments. Like fungal DHN-melanin and sepia melanin (40), the melanized bacteria in this study could be repeatedly engaged in redox-cycling reactions that yielded amplified output currents, which indicated that melanin can be reversely and stably switched between oxidized and reduced redox states. This property will allow bacterial cells to exchange electrons with diffusible redox-active species in the cellular environment and sense biological or environmental redox signals. Moreover, it has been reported that the biological melanin may perform energy-harvesting activities (41, 42) using an unknown mechanism. Thus, the melanized bacteria potentially can be developed as living materials to preserve charge storage capacity and power transient electrical devices.

To test the photoprotective property of bacterial melanin, we irradiated bacterial
cells with short-wavelength UVC, which is the most damaging type of UV radiation. UVC is absorbed by DNA and results in the formation of pyrimidine adducts and strand breaks (43). Initially, we irradiated the PBS-washed melanized bacterial cells with UVC and did not see improved UV resistance compared with that with the nonmelanized cells. This finding suggested that the melanin granules deposited in the cell wall and cytoplasm were not sufficient to absorb UV radiation. When melanized cell cultures were irradiated with UVC, cells grown in the melanin-containing medium showed significantly higher resistance (Fig. 6A). This result was similar to findings from our previous report that pyomelanin-containing supernatant from *Vibrio campbellii ΔhmgA* culture demonstrated protective property against oxidative stress (44). Melanin is known to scavenge reactive oxygen species (ROS) generated by UV in solution, and its scavenging capability is proportional to its concentration (32). Therefore, our result indicated that the larger volume of melanin particles in the cell medium greatly improved melanin’s ability to attenuate UVC penetrating into cells and scavenge ROS generated from irradiation in the aqueous environment. The photoprotective property of MNPs was further confirmed by applying the filtered supernatant to HeLa cells (Fig. 6B). Therefore, large-scale production of MNPs from *V. natriegens* may have potential to make cost-effective photoprotection materials.

Melanin has been known for its affinity to adsorb various chemicals and drugs, including harmful substances, which results in protection of pigmented cells and tissues (45). Gustavsson et al. (13) successfully demonstrated that *E. coli* cells coated with melanin were able to adsorb up to 80% of the antimalarial drug chloroquine at concentrations typical for pharmaceutical pollution in wastewater. Here, we showed that the melanized marine bacterium was able to robustly sequester the explosive compound TNT from an aqueous solution. The discharge of explosive compounds such as TNT from explosive manufacturing and unloading plants to wastewater has been of increasing concern. Currently, TNT-contaminated water is treated with granular activated carbon (GAC) adsorption (46). However, the regeneration of GAC may be an issue when a large amount of TNT is adsorbed. Our study demonstrates that melanin-producing *V. natriegens* is able to provide dynamic and economic TNT-adsorbing materials that may be regenerated through changes in pH.

In summary, we successfully engineered a *V. natriegens* strain to rapidly and economically produce melanin from tyrosinase, and analyses revealed that this melanin exhibited physical and chemical properties similar to those of natural and chemically synthesized melamins. We anticipate that producing and controlling melanin structures at the nanoscale in this fast-growing bacterial system with synthetic biology tools will lead to the generation of novel biomaterials, including protection of chemical and radiation threats, therapeutics, electronics, sensing, and bioremediation that could benefit both military and civilian populations.

**MATERIALS AND METHODS**

**Strains and growth conditions.** *V. natriegens* strain ATCC 14048 was grown in LBv2 (Luria broth [Miller] supplemented with 204 mM NaCl, 4.2 mM KCl, and 23.14 mM MgCl₂) or M9 medium (11.28 g M9 minimal salts H₁₁₀₀₃, 4 g glucose, 15 g NaCl, 0.5 g MgSO₄, 10 mg CaCl₂, and 40 mg CuSO₄ per liter), unless otherwise stated. Tripeptides were purchased from Peptide2.0, Inc. (Chantilly, VA). HeLa ATCCCCL-2 cells (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle medium (DMEM; Thermo Fisher Scientific, IL).

**Construction of melanin-producing *V. natriegens*.** The tyrosinase gene from *Bacillus megaterium* Tyr1 was synthesized by Eurofins Genomics (Louisville, KY), similar to a previous study (13). The PCR-amplified *tyr1* sequence was cloned into the plasmid pJV298 under the control of the inducible promoter Pₜₐ₅ (26), replacing the green fluorescent protein (GFP) gene with the Gibson Assembly master kit (New England BioLabs, Ipswich, MA) with four primers, as follows: tyr1-1, ATGTATATCTCCTTAAGCTTACG; tyr-2, TGAGGATCCGGTGATTGATTG; vect-1, GCTTAAGGAGATATACATATGGTAACAAGTATAGAGTTAGAAAAAAC; and vect-2, AATCACCGGATCCTCATGAGGAACGTTTTGATTTTC (lowercase letters complement the vector sequence, and uppercase letters complement the *tyr1* sequence), and a Gibson Assembly reaction was set up to insert the PCR product into the digested
Melanin Production in *Vibrio natriegens*

**Melanin production.** *V. natriegens/pJV-Tyr1* from a glycerol stock was inoculated into 3 ml LBv2 liquid medium supplemented with 6 µg/ml chloramphenicol (LBv2-Cm) and grown at 30°C overnight. A 0.5-ml aliquot of the overnight culture was transferred into 50 ml LBv2-Cm medium and incubated at 200 rpm at 37°C for 2 h. Tyrosinase production was induced by the addition of 200 µM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for three more hours. Cells were pelleted from the culture via centrifugation, washed with M9 minimal medium twice, and resuspended in 50 ml M9 medium supplemented with 50 µg/ml CuSO₄ and 0.4 mg/ml L-tyrosine (nonmelanized bacterial cells were prepared by omitting L-tyrosine in the same medium). The melanized bacterial cells were harvested by centrifugation, and the black supernatant that contained melanin nanoparticles was passed through a Millipore 0.2-µm polycarbonate membrane to remove cell debris. Melanin was precipitated from the supernatant by adding a 1/10 volume of 6 M HCl, washed with deionized water until neutral pH, and lyophilized into black powder. Bacterial growth curves were measured at an optical density at 600 nm (OD₆₀₀) using a Bioscreen C analyzer (Growth Curves USA, Piscataway, NJ). Pigment intensities from melanized bacterial cultures (adding L-tyrosine in the medium) were measured at an OD₆₀₀ by subtracting readings from nonmelanized bacterial cultures (omitting L-tyrosine in the same medium) using the same instrument. OD₆₀₀ values were converted to melanin yields using the standard curve made from the melanin powder precipitated from the supernatant. The rate of melanin production was calculated as the quantity of melanin produced and divided by the estimated cell dry weight in 1 ml of culture within a 1-h period before melanin saturation (14). The immunofluorescent analysis method for the melanized cells is described in the supplemental material.

**Proteomic quantification of the expressed tyrosinase.** IPTG-induced and -uninduced *V. natriegens/pJV-Tyr1* cultures were normalized to the same cell numbers after counting with the flow cytometer. Cell pellets were resuspended in 100 µl of 10% n-propanol in 50 mM ammonium bicarbonate (NPABC). Thirty microliters of the cell suspension was lysed, digested with trypsin in a Barocycler (Pressure Biosciences, Inc.), dried in the speed vacuum, and further suspended in 90 µl of 0.1% formic acid. Three microliters of this suspension was injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system for analysis (Thermo U3000 nano-LC coupled to a Thermo Orbitrap Fusion Lumos mass spectrometer). The acquired data were converted into mgf files and searched with Mascot against a *Vibrio natriegens* database, custom database containing the tyrosinase sequence, and common contaminant database. Selected peptides assigned to the tyrosinase protein and DnaK were then manually extracted from the raw data, and the chromatographic peak area was calculated. Area ratios between induced and uninduced samples are reported in Table 1. The DnaK protein was used as a representative of endogenous protein not controlled by the inducer.

**Generation and characterization of melanin ghosts.** The protocol for making bacterial melanin ghosts was modified from a method previously developed in fungi (31). Melanized bacteria were incubated with 4 M guanidinium thiocyanate at room temperature (RT) for 10 min. Cells were washed with deionized water once and hydrolyzed by boiling in 6 M HCl for 20 min. The resulting black particles (melanin ghosts) were washed with deionized water multiple times until a pH of 6 was obtained. Imaging of the melanin ghosts and melanized *V. natriegens* cells was conducted using a Philips CM200 TEM and a FEI Quanta 600 SEM, respectively.

**Sample preparation for imaging.** Bacterial cells or melanin ghosts were prefixed with 2.5% glutaraldehyde, 100 mM HEPES buffer, 50 mM L-lysine, and 7.5% ruthenium red for 30 min, fixed with the same solution minus L-lysine for 2 h, and postfixed with a 4% osmium tetroxide (OsO₄) solution for 2 h. Each sample was dehydrated in increasing amounts of acetone (25%, 50%, 70%, 95%, and 100%) for 30 min at each step. TEM samples were trimmed and ultramicrotomed using an RMC Ultracut microtome with a 35° DiaTome diamond knife. Sections were cut at a thickness of 70 nm and picked up onto 400-mesh Cu grids. Once the grids were dried, they were stained with UranylLess stain.

**Measurement of melanin nanoparticles.** Dynamic light scattering (DLS) measurements were carried out using a Zetasizer Nano series instrument equipped with a HeNe laser source (λ = 633 nm) (Malvern Instruments Ltd., Worcestershire, UK) and analyzed using dispersion technology software (DTS) (Malvern Instruments Ltd.). Structural characterization of melanin nanoparticles was carried out using a JEM-2100 TEM. Samples for TEM were prepared by spreading a drop (5 to 10 µl) of melanin nanoparticles onto ultrathin carbon/holey support film on a 300-mesh Au grid (Ted Pella, Inc.) and letting it dry. Individual particle sizes were measured using a Gatan digital micrograph (Pleasanton, CA); average sizes along with standard deviations were extracted from an analysis of ~100 nanoparticles.

**Fourier transform infrared spectroscopy.** One milligram of melanin powder was ground with 160 mg anhydrous potassium bromide (KBr; FTIR grade, ≥99% trace metals basis; Sigma-Aldrich) and compressed into a semitransparent pellet using a hydraulic press (Omega CN9000). The transmission FTIR measurements (iS50 FTIR; Nicolet) were taken with an air background.
Electron paramagnetic resonance. Melanin powder, melanin ghosts, and dried, melanized bacteria were characterized by EPR at 300 K in a Bruker 9.5-GHz spectrometer. Typical microwave powers of 5 to 20 mW with 1-G modulation amplitude and 100 kHz field modulation were employed for these experiments. EPR spectra were also obtained for the melanin samples after illumination with a xenon (Xe) lamp (75 W, 350 to 1,000 nm wavelength) for 30 min at RT.

Electrochemical measurements. Melanized and nonmelanized V. natriegens cells were probed for redox activity by first immobilizing them within a Ca²⁺-alginate hydrogel film at an electrode surface. These films were prepared by electrodeposition by suspending the cells in a mixed solution containing 1% alginate and 0.25% CaCO₃ particles and electrodepositing the film onto a standard gold electrode by immersing this electrode into the mixed solution and biasing it to serve as the anode (4 A/m², 60 s), using a Pt wire as the cathode (2400 SourceMeter; Keithley) (47, 48). Electrochemical probing for the redox activity of these films was performed using cyclic voltammetry (CV) in a three-electrode system (CHI Instruments 600C electrochemical analyzer), as follows: (i) the film-coated standard gold electrode was the working electrode, (ii) Ag/AgCl was the reference electrode, and (iii) Pt wire was the counterelectrode. Air was excluded by purging N₂ during the experiment. All of the CV experiments were performed at the scan rate of 2 mV/s.

Cell survival assay. Twenty microliters of the nonmelanized and the melanized V. natriegens cell cultures or washed cells suspended in PBS was irradiated with UVC (254 nm) at 450 ml/cm² for 20 min, diluted with PBS, and spotted on LBv2 agar plates. HeLa cells were seeded on 35-mm dishes with 14-mm glass-bottom inserts (no. 1.0 cover glass; MatTek Corp., MA, USA) at a density of ~7 × 10⁴ cells/ml (3 ml/well) and incubated under regular cell culturing condition (5% CO₂ at 37°C) for 24 h to a confluence of 70 to 80%. Dishes were coated with fibronectin (10 to 20 μg/ml) in Dulbecco’s phosphate-buffered saline (DPBS) before adding the cell suspension. The cell monolayers on the dishes were washed with DPBS three times and submerged with the supernatant filtered from the melanized V. natriegens culture diluted (1:1) with live cell imaging solution (LCIS). Cells were then subjected to UV irradiation (254 nm, 6 ml/cm²) for 10 s, followed by incubation for 24 h in under regular culturing medium and conditions. Next, cells were washed with LCIS, followed by staining with a fluorescence LIVE/DEAD viability kit (Thermo Fisher Scientific, Grand Island, NY) for live/dead quantification. The stained cells were imaged with confocal laser scanning microscopy (CLSM) using a Nikon A1Rsi confocal microscope. Fluorescence images of individual cells were counted and quantified for live (green) and dead (red) status for 50 to 70 hundred microliters of cells in this buffer was then mixed with a solution containing 125 ppb TNT in the same buffer with the appropriate pH. This mixture was vortexed for 30 min before pelleting by centrifugation. The concentration of TNT in the supernatant was measured using an Agilent 1290 Infinity high-performance liquid chromatography (HPLC) system equipped with an Agilent reverse-phase C₁₈ analytical column (Eclipse XDB-C₁₈; 5 mm; 4.6 by 250 mm²; Santa Clara, CA, USA). UV-Vis detection at 254 nm was performed to monitor the elution of TNT.

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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