

Isolation of Antibiotics Turbomycin A and B from a Metagenomic Library of Soil Microbial DNA

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To access the genetic and biochemical potential of soil microorganisms by culture-independent methods, a 24,546-member library in *Escherichia coli* with DNA extracted directly from soil had previously been constructed (M. R. Rondon, P. R. August, A. D. Bettermann, S. F. Brady, T. H. Grossman, M. R. Liles, K. A. Loiacono, B. A. Lynch, I. A. MacNeil, M. S. Osburne, J. Clardy, J. Handelsman, and R. M. Goodman, *Appl. Environ. Microbiol.* 66:2541–2547, 2000). Three clones, P57G4, P89C8, and P214D2, produced colonies with a dark brown melanin-like color. We fractionated the culture supernatant of P57G4 to identify the pigmented compound or compounds. Methanol extracts of the acid precipitate from the culture supernatant contained a red and an orange pigment. Structural analysis revealed these were triaryl cations, designated turbomycin A and turbomycin B, respectively; both exhibited broad-spectrum antibiotic activity against gram-negative and gram-positive organisms. Mutagenesis, subcloning, and sequence analysis of the 25-kb insert in P57G4 demonstrated that a single open reading frame was necessary and sufficient to confer production of the brown, orange, and red pigments on *E. coli*; the predicted product of this sequence shares extensive sequence similarity with members of the 4-hydroxyphenylpyruvate dioxygenase (4HPPD) family of enzymes. Another member of the same family of genes, *lly*, which is required for production of the hemolytic pigment in *Legionella pneumophila*, also conferred production of turbomycin A and B on *E. coli*. We further demonstrated that turbomycin A and turbomycin B are produced from the interaction of indole, normally secreted by *E. coli*, with homogentisic acid synthesized by the 4HPPD gene products. The results demonstrate successful heterologous expression of DNA extracted directly from soil as a means to access previously uncharacterized small organic compounds, serving as an example of a chimeric pathway for the generation of novel chemical structures.

Microorganisms cultured from soil have provided most of the antibiotics and many other medicinal agents that have dramatically improved human health in the latter half of the 20th century. Despite this fruitful history, traditional searches for new natural products from cultured soil microorganisms are now confronting diminishing returns for the discovery of new compounds; the rate of rediscovery of known antibiotics approaches 99.9% (48). In light of the need for new antibiotics to combat the multidrug-resistant pathogens that have recently emerged, new approaches to antibiotic discovery are needed. One of the richest sources of new antibiotics may be the uncultured microorganisms of soil.

The number of microorganisms typically cultured from soil represents 1% or fewer of the total microbial community (44, 45). DNA-DNA reassociation measurements and other culture-independent methods reveal that the total genetic diversity in a soil sample of 100 g or less is likely between 4,000 (44) and 13,000 species (45). Recent analyses of 16S rRNA genes amplified directly from soil indicated that novel phyla of *Bac-*

teria and *Archaea* are present (2, 3, 16, 21, 22, 26, 41). If the diversity of chemistry produced by the culturable bacteria is an indicator of the chemical capacity of the uncultured bacteria, then many molecules, and perhaps useful drugs, remain to be discovered from soil microorganisms.

The challenge inherent in exploiting the uncultured microorganisms in soil for drug discovery is gaining access to their metabolites without relying on traditional culturing methods. It was proposed that these metabolites be accessed by a direct cloning of soil DNA into a bacterial artificial chromosome (BAC) vector, thereby accessing the collective genomes, or “metagenome,” from soil microorganisms (17, 33). A number of reports indicate that this approach is technically feasible and can reveal novel biology and chemistry, including novel antibiotics (4, 5, 19, 20, 32, 42, 46).

A 24,546-member metagenomic library of DNA extracted from soil had previously been constructed (32). Here we report the isolation and characterization of two colored triaryl cation antibiotics that are produced in *Escherichia coli* at elevated levels by members of this library.

MATERIALS AND METHODS

Strains, library construction, and screening. *E. coli* strain DH10B [F *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*dlacZ*ΔM15 Δ*lacX74* *deoR* *recA1* *endA1* *araD139* Δ(*ara*, *leu*)7697 *galU* *galK* λ *rpsL* *nupG*] was used for all cloning and culturing. The library was constructed in the vector pBeloBAC11, as previously described

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(32). Chloramphenicol was added to all media at 12.5 µg/ml. Hemolytic activity was detected on blood agar plates containing 7% defibrinated sheep blood in Luria-Bertani (LB) agar. Color production was observed by visual screening of colonies on LB agar.

Cloning and transposon mutagenesis. We digested DNA isolated from clone P57G4 with *Pst*I and cloned the products into pGEM-5Zf+ (Promega, Madison, Wis.) following standard techniques (38). The GPS-1 Genome Priming System (NEB; Beverly, Mass.) was used to generate **transposon insertions** according to the manufacturer's instructions. Donor plasmid and target DNA were mixed at a 2:1 molar ratio, and transformants were selected on LB plates containing chloramphenicol and kanamycin (20 µg/ml).

DNA sequencing and analysis. DNA sequencing was performed at the University of Wisconsin—Madison Biotechnology Center on an ABI Model 377 automated sequencer. Sequence similarity searches were conducted with BLAST (1), and other gene sequence analyses were conducted with DNASTAR software (Madison, Wis.).

Construction of a clone expressing the legiolsin (*lly*) gene. *lly*-specific primers were designed based on sequences in GenBank (accession no. AF075724) and used to amplify the *lly* coding sequences and upstream promoter regions from *Legionella pneumophila* genomic DNA according to standard protocols (37, 47). The PCR product was cloned into the vector pGEM-T (Promega). Clones containing the *lly* gene were verified by restriction digestion and homogentisic acid (HGA)-melanin pigment production.

Characterization of colored compounds and TLC analysis. Cell-free culture broth from P57G4 grown in LB medium for 48 h at 37°C was adjusted to pH 13.0 with NaOH, stirred for 1 h, and then acidified to pH 2.0 with HCl. The methanol extract of the precipitate that was collected by filtration after 24 and 72 h was resuspended in chloroform-methanol (80:20) and passed over a silica plug to remove any remaining insoluble material. Cell-free culture broth was also extracted with ethyl acetate (volume equal to that of the culture broth). The ethyl acetate fraction was washed with water and dehydrated with NaCl-saturated water prior to evaporation. Samples were resuspended in ethyl acetate for analysis. Silica gel thin-layer chromatography (TLC), with MetOH-CHCl₃ (1:9) as a solvent, was used to analyze culture production of colored compounds. For further details about chemical analysis, see the following website: <http://www.plantpath.wisc.edu/fac/joh/turbomycin>.

Chemical synthesis of turbomycin A and B. The triaryl methanes of turbomycin A and B were made by heating 250 µmol of the appropriate aldehyde (indole-3-carboxyaldehyde for turbomycin A and benzaldehyde for turbomycin A), 500 µmol of indole, 40 µl of acetic acid, and 360 µl of ethanol at 80°C for 4 h (6). The reaction was neutralized with 10% NaOH, and the triaryl methane was oxidized in situ by addition of 200 mg of tetrachloro-1,4-benzoquinone and heating at 80°C for 40 min. An equal volume of 10% NaOH was then added, and the reaction was extracted three times with ethyl acetate to obtain a crude reaction product. Synthetic turbomycin A, spectroscopically identical to the natural product, was obtained from the crude indole-3-carboxyaldehyde reaction product following two normal-phase chromatography steps (~~chloroform-methanol-triethylamine [90:10:0.1] followed by chloroform-methanol-acetic acid [80:20:0.1]~~). Synthetic turbomycin B, spectroscopically identical to the natural product, was obtained from the crude benzaldehyde reaction product following two normal-phase chromatography steps (chloroform-methanol-triethylamine [98:2:0.1] followed by chloroform-methanol-acetic acid [90:10:0.1]).

Cell-free assay for triaryl cation production. A range of HGA, indole, and indole-3-carboxyaldehyde concentrations (1 µM to 1 mM) was added to 10 ml of LB medium, separately and in combination, followed by incubation at 37°C in aerated 18-mm-diameter culture tubes for 48 h. The cell-free medium was then adjusted to pH 13 with NaOH, stirred for 1 h, and acidified to pH 2 with HCl for 48 h at room temperature or directly acidified to pH 2. The two procedures produced identical precipitates. The dark brown precipitate was collected by centrifugation, extracted with methanol, and resolved by TLC as described above.

Assays for inhibition of microbial growth. **Target organisms included** *E. coli* (HS997), *Staphylococcus aureus* (strain 3001), *Enterococcus faecalis* (strain 4), *Streptococcus pyogenes* (strain 8P01), *Pseudomonas aeruginosa* (strain 9020), *Bacillus subtilis* (strain BR151pPL608), *Bacillus cereus* subsp. *mycoides* (strain 1003), *Erwinia herbicola* (strain IRO), *Salmonella enterica* serovar Typhimurium (strain LT2), *Streptomyces griseus* (strain 6501) and *Candida guilliermondii* (strain Y001). Screening plates were prepared by diluting an overnight brain heart infusion (BHI) broth culture (1:1,000 dilution) in yeast extract (YE) and spreading 0.1 ml of this mixture (BHI-YE) on an agar plate. Natural products and synthetic compounds, dissolved in dimethyl sulfoxide (DMSO) to a concentration of 250 µg/ml, were applied as 10-µl drops to the surface of the plate. The plates were placed at 4°C overnight (12 h) and then were incubated at either 37°C

(*E. coli*, *S. aureus*, *E. faecalis*, *S. pyogenes*, and *P. aeruginosa*) or 28°C (*B. subtilis*, *B. cereus*, *E. herbicola*, *S. enterica* serovar Typhimurium, *S. griseus*, and *C. guilliermondii*) until a lawn developed. Inhibition was scored visually, and zones of inhibition were reported as the diameter of the clear zone in millimeters. The assay was conducted on duplicate plates.

Pythium ultimum (strain 1033) and *Fusarium solani* f. sp. *glycine* (strain 90.1) were grown on one-quarter-strength potato dextrose agar (11) at 23°C. Plugs were cut from the fungal lawn using a no. 5 cork borer and placed on the center of a fresh plate containing the compounds dissolved in DMSO as above. The plates were incubated 7 days or until the organism covered the plate at 23°C in the dark.

To determine the MICs of the antibiotics, overnight cultures were prepared as described above. The optical densities (OD) of the cultures were normalized to 0.15 (150-µl path length) by performing 1:1 serial dilutions in a 96-well microtiter plate. Inoculum cell concentrations were subsequently determined by serial dilution and plating on BHI-YE medium.

Test compounds, both natural product and synthetic, were dissolved in DMSO (2.0 mg/ml) and diluted into BHI-YE medium to a concentration of 106 µg/ml (DMSO, 5.35% [vol/vol]). One hundred fifty microliters of each of the test compounds was added to three separate wells, each containing 150 µl of BHI-YE medium (no DMSO). Serial dilutions (1:1) were performed (150-µl transfer into 50 µl of diluent containing 2.6% DMSO) to achieve final concentrations of 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 µg/ml. Three plates were prepared for each test organism (three plates with three replicates per plate, giving a total of nine per compound per organism). Additional wells were included in duplicate on each plate for color subtraction, with or without DMSO, and minus compound controls (three plates with two replicates per plate, giving six per control per organism).

Ten microliters of normalized inoculum was added per well. Absorbance at 590 nm was read by use of a Wallac Victor Microtiter plate reader; double circular mixing was performed for 10 s prior to reading at times 0, 30, and 60 h. The plates were incubated statically at 28°C when not being read. Absorbance values were imported into Microsoft Excel and analyzed graphically.

RESULTS

Identification of pigment-producing clones in soil DNA library. We screened a 24,546 member library (SL2) of DNA from soil cloned into a BAC vector. The average insert size within this library is 44.5 kb (32). **Screening for hemolytic activities initially identified one clone, designated P57G4, that produced a zone of clearing on blood agar and also produced dark brown and orange colors when grown in LB medium.** The color of P57G4 prompted a closer examination of the culture broth. Subsequent analysis of additional hemolytic clones from SL2 revealed two additional brown-pigmented clones, designated P89C8 and P214D2, which exhibited properties similar to those of P57G4 in solid and liquid culture.

Characterization of the acid precipitate. The presence of a dark brown color in bacterial cultures is characteristic of the production of melanin or a melanin-type polymer that can be collected from culture broth by acid precipitation (15). Upon acidification of the P57G4 culture broth, a dark brown precipitate formed, suggesting the presence of melanin in the culture. The dark brown material in the acid precipitate harvested from the P57G4 broth, like other bacterial melanins (15, 39), was insoluble in aqueous acid, was soluble in aqueous base, bound to an anion-exchange column (DEAE-cellulose), and was >7,000 Da.

Antibiotic isolation and characterization. We examined the acid precipitate from P57G4 cultures for additional colored compounds that could be extracted with an organic solvent. **Two colored compounds were extracted with methanol from the acid precipitate that formed when the cell-free culture broths of P57G4, P89C8, and P214D2 (grown in LB for 48 h at 37°C) were brought to pH 13 with NaOH, stirred for 1 h, and**

TABLE 1. Antibiotic activities of synthetic and natural turbomycins A and B

Organism and strain	Growth inhibition ^a by:			
	Turbomycin A		Turbomycin B	
	Synthetic	Natural	Synthetic	Natural
Gram-negative bacteria				
<i>E. herbicola</i> IRQ	+	+	+	+
<i>E. coli</i> HS997	+/-	+/-	+/-	+/-
<i>P. aeruginosa</i> 9020	-	-	-	-
<i>S. enterica</i> serovar Typhimurium LT2	+	+	+	+
Gram-positive bacteria				
<i>B. cereus</i> subsp. <i>mycooides</i> 1003	+	+	+	+
<i>B. subtilis</i> BR151pPL608	+	+	+	+
<i>E. faecalis</i> 4025	-	-	-	-
<i>S. aureus</i> 3001	+	+	+	+
<i>S. pyogenes</i> 8P01	+	+	+	+
<i>S. griseus</i> 6501	+	+	+	+
Fungi or Protists				
<i>C. guilliermondii</i> Y001	+/-	+/-	+/-	+/-
<i>P. ultimum</i> 1033	-	-	-	-
<i>F. solani</i> (f. sp. <i>glycine</i>) 90.1	-	-	-	-

^a +, 2- to 13-mm zone; +/-, 1-mm zone, -, no zone of inhibition.

then acidified to pH 2 with HCl. Using normal-phase chromatography, an orange compound (turbomycin A) and a red compound (turbomycin B) were isolated in approximately a 20:1 ratio from the methanol extract. Both compounds show broad-spectrum antimicrobial activities (Table 1) but did not exhibit hemolytic activity when spotted on blood agar.

High-resolution fast atom bombardment mass spectrometry (HRFABMS) data for turbomycin A indicated a molecular formula of C₂₅H₁₈N₃, while 1-D and 2-D nuclear magnetic resonance (NMR) experiments suggested the presence of only five protons and nine carbons. The ortho-substituted aromatic ring of the indole was easily identified from the 4-carbon spin system seen in ¹H-¹H RelayH experiments, and the nitrogen at position 1 was suggested by the deshielding of C-2, C-7a, and H-2. The remainder of a C-3 substituted indole was confirmed by ¹H-¹³C heteronuclear multiple bond correlations from C-3a, C-3, C-7a, and C-8 to H-2. All five protons and nine carbons observed by NMR are present in the C-3 substituted indole partial structure. The structure of turbomycin A as the indole trimer shown in Fig. 1 was supported by the molecular formula deduced by HRFABMS. However, we were not able to conclusively establish this structure using spectral data alone. Therefore, synthesis and single crystal X-ray diffraction analysis of the synthetic material were used to confirm the trimeric structure. The triindole methane was made by heating indole-3-carboxyaldehyde and indole in 10% acetic acid and then oxidized in situ with tetrachloro-1,4-benzoquinone to yield an orange compound that was spectroscopically identical to the natural product. The structure of the synthetic material was confirmed by single crystal X-ray diffraction analysis of the perchlorate salt (Fig. 1).

In liquid culture, the MIC of synthetic turbomycin A (orange) was 6.2 μg/ml for *E. herbicola*, *B. subtilis*, *S. aureus*, and *S. pyogenes* and 12.5 μg/ml for *S. enterica* serovar Typhimurium.

The natural product and synthetic compound were tested in the same experiment against *S. aureus* and had the same MIC.

For turbomycin B, the presence of the indole was deduced in the same manner as described above for turbomycin A and the phenyl group was apparent from both the ¹H NMR spectrum and the additional spin system observed in ¹H-¹H RelayH experiments. Again, the trimeric structure shown in Fig. 1 was suggested by the molecular formula predicted by HRFABMS, C₂₃H₁₇N₂. As with turbomycin A, the proposed structure of turbomycin B was confirmed by synthesis; benzaldehyde was heated with indole in 10% acetic acid, and the resulting heterotrimeric methane was then oxidized in situ with tetrachloro-1,4-benzoquinone to yield a red compound that was spectroscopically identical to the compound isolated from the P57G4 culture media.

Turbomycin A has been previously characterized from a fungal source (6) and synthesized (6, 43); however, it has never been reported as a bacterial metabolite. Turbomycin B has not been previously described as a natural product.

Genetic characterization of the antibiotic-producing clones.

The P57G4 clone contains a 25-kb insert of DNA. *Pst*I fragments of P57G4 were subcloned into pGEM-5Zf+ to generate templates for end sequencing. One of the subclones, pDG1, produced a brown pigment when grown in liquid and on solid medium. TLC analysis of supernatant precipitates from P57G4 and pDG1 cultures revealed that both turbomycin A and turbomycin B were present in pDG1 cultures, indicating that the genetic information required for color production in P57G4 cultures was present on the pDG1 subclone. pDG1 contains a 3-kb insert entirely from the P57G4 environmental insert DNA. Sequence analysis of this insert revealed three open reading frames (ORFs).

Clone pDG1 was mutagenized with transposon GPS-1, and the resulting mutants were screened for color production. The five non-color-producing insertional mutants all carried the transposon within the ORF1 coding sequence. Of six color-producing insertional mutants, two carried an insertion in the vector and four carried an insertion in nonvector sequences outside the ORF1 coding sequences. TLC analysis indicated that clones carrying insertions within ORF1 produced no observable turbomycin A or turbomycin B (data not shown).

These results suggest that ORF1 is necessary and sufficient for color production in *E. coli*.

The ORF1 nucleotide sequence is 58% G+C. The deduced protein encoded by ORF1 is 40 kDa, containing 353 amino acids. The predicted protein shares extensive similarity with

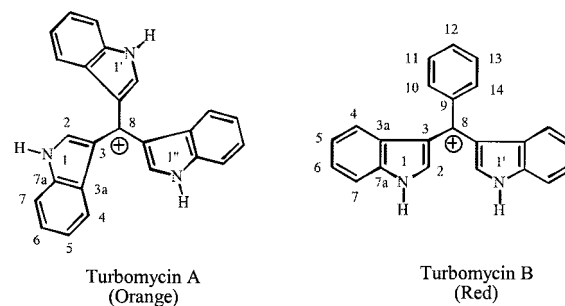


FIG. 1. Chemical structures of turbomycin A and turbomycin B.

legiolysin (Lly) in *L. pneumophila* (Q53407) (53% identity, 71% similarity) (47), hemolysin (Vlly) in *Vibrio vulnificus* (U31553) (54% identity, 71% similarity) (8), 4-hydroxyphenylpyruvate dioxygenase (4HPPD) in *Pseudomonas* sp. (P80064) (49% identity, 67% similarity) (34), and MelA in *Shewanella colwelliana* (P23996) (45% identity, 63% similarity) (14).

P89C8 and P214D2 contain inserts of 80 and 45 kb, respectively. Sequence analysis of non-color-producing transposon mutants of P89C8 and P214D2 revealed additional sequences encoding translation products with similarity to members of the 4HPPD family. Both of the predicted proteins are more closely related to the *Pseudomonas* sp. family member (97% identity, 98% similarity) than to Lly (47% identity, 65% similarity). The G+C contents of P89C8 and P214D2 sequences (55% and 53%, respectively) are also lower than P57G4 sequences.

Additional sequencing of subclones from P57G4 insert DNA identified a gene sequence with homology to homogentisate deoxygenase (*hmgA*). *HmgA* degrades HGA in organisms that are believed to utilize the 4HPPD gene product as part of the tyrosine degradation pathway (13, 28), thereby preventing the formation of HGA-melanin. Translation and analysis of the remaining ORFs in pDG1 revealed deduced proteins containing similarity to other putative members of the tyrosine degradation pathway, suggesting that the soil microorganism represented by clone P57G4 possesses the biochemical capacity for both HGA synthesis and degradation and likely does not produce HGA-melanin. Sequences flanking the 4HPPD homologs in BACs P89C8 and P214D2 do not indicate that these genes are integrated within a similar degradation pathway operon in the original host genome.

Characterization of antibiotic production. The methanol extract from the uninoculated LB medium control, treated in the same manner as P57G4 cultures, did not contain turbomycin A or B when analyzed by TLC. However, the methanol extracts of the acid precipitate harvested from control cultures (*E. coli* transformed with the BAC vector containing no insert DNA) contained small quantities of both turbomycin A and turbomycin B. The major colored metabolite turbomycin A was present in up to 80-fold excess in the culture of P57G4 over that present in the BAC vector control. The minor metabolite, turbomycin B, did not show as significant an increase in the clone when compared with the BAC vector control. Methanol extracts of acid precipitates from both the P57G4 clone and the vector control contained increasing amounts of both compounds with longer periods of incubation at reduced pH. We addressed the possibility that turbomycin formation is dependent on acidification by extracting culture supernatants directly with ethyl acetate and analyzing extracts for turbomycin A production. Our results were similar to the methanol extract results, confirming that acidification is not required for turbomycin A production. Although the origin of the turbomycin compounds requires further clarification, rapid accumulation appears to be dependent on two sources, one endogenous to the *E. coli* host and one resulting from heterologous expression of the 4HPPD enzyme.

We tested whether a related 4HPPD enzyme could catalyze triaryl cation production by introducing the *L. pneumophila* *lly* gene into *E. coli*. Clones containing *lly* also produced a melanin-like brown pigment and had elevated levels of triaryl cat-

ions in the acid precipitate harvested from the culture broth (data not shown), strongly suggesting that the formation of triaryl cations is associated with the presence of HGA-melanin in the culture medium.

We next evaluated the potential for synthetic HGA alone to catalyze triaryl cation production in a cell-free system, utilizing both methanol and ethyl acetate extraction protocols. The addition of 1 mM HGA to LB medium resulted in the formation of significant quantities of HGA-melanin pigment by 48 h. However, the formation of HGA-melanin from HGA (1 μ M to 1 mM) in our cell-free system was not sufficient for the production of the orange or red pigments. The addition of indole (1 mM), which is secreted at high levels by *E. coli* (40), did result in the formation of small quantities of both pigments in LB medium. As was observed with the HGA-melanin produced as a result of introducing ORF1 or *lly* into *E. coli*, the addition of HGA (1 mM) to LB medium containing indole (1 mM) resulted in a substantial increase in the formation of both a red compound and an orange compound that comigrated with turbomycin A and B.

DISCUSSION

We have demonstrated the direct cloning and heterologous expression of environmentally derived DNA as a means to access new biologically active small organic compounds. We isolated and characterized two broad-spectrum triaryl cation antibiotics present at elevated levels in three color-producing clones from a BAC library containing soil DNA. Sequence analysis of P57G4 identified a bacterial locus containing three putative members of a tyrosine degradation pathway, with an organization similar to that of a *Sinorhizobium meliloti* locus. A single ORF from P57G4, related to the 4HPPD family, was necessary and sufficient to cause the high-level production of triaryl cations and a melanin-type pigment in *E. coli*. Sequence analysis of P89C8 and P214D2 revealed additional members of the 4HPPD family, more closely related to previously reported sequences than is the P57G4 predicted product. Transposon mutagenesis confirmed that these 4HPPD genes enhanced hemolytic and pigment-producing activities in the *E. coli* host cell.

Lly, Vlly, MelA, and 4HPPD are all members of the 4HPPD family that catalyze the production of HGA. Of the bacteria in which a 4HPPD gene has been identified, two classes are apparent. In one class, which includes *L. pneumophila* and *Vibrio cholerae*, expression of 4HPPD family members results in the accumulation of HGA-melanin, a dark pigment generated by the spontaneous polymerization of HGA under aerobic conditions (9, 14, 47). This polymerization product is a complex polymer that has been shown to protect against light damage in *L. pneumophila* and to avidly bind redox-active metals (25). The spontaneous oxidation and polymerization of HGA also leads to hemolysis in the presence of red blood cells (18).

The second class of bacteria, which includes *S. meliloti* (28) and *Streptomyces coelicolor* (31), expresses the 4HPPD gene but has not been shown to produce HGA-melanin. These bacteria are believed to be utilizing the 4HPPD gene product as part of the tyrosine degradation pathway, depending on the activity of *HmgA* to metabolize HGA. Mutations in *hmgA* in *S. meliloti* result in the accumulation of a brown pigment, likely HGA-melanin (28). Similar observations have been made with

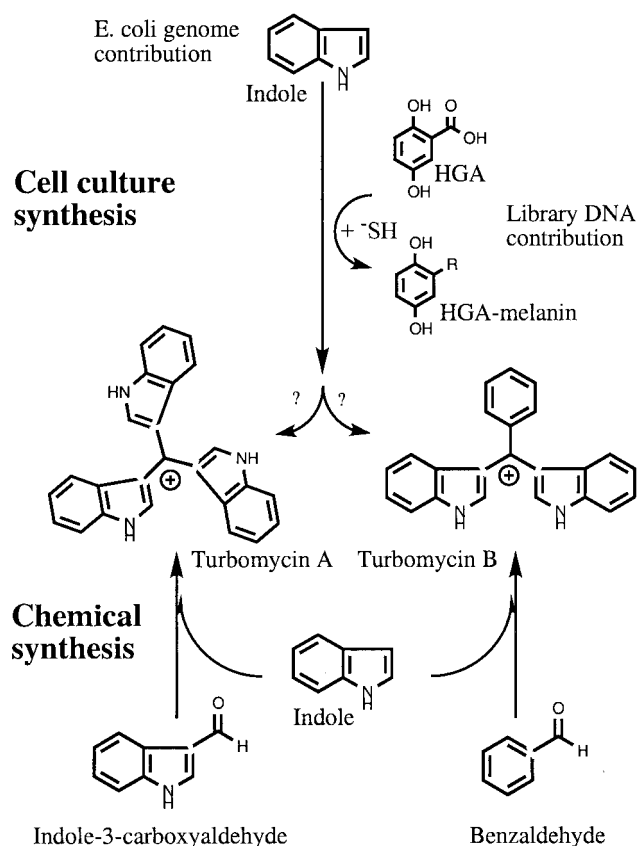


FIG. 2. Proposed mechanisms of turbomycin A and B synthesis. The oxidative polymerization of HGA to HGA-melanin serves as the catalyst for the formation of turbomycin A and B, utilizing unidentified molecules as donors for the central carbon and the benzyl group. The aldehyde carbon is believed to be the central carbon donor in chemical synthesis.

Aspergillus nidulans, which carries a mutation in its *hmgA* gene (13).

In an oxidative tyrosine degradation pathway, 4HPPD catalyzes the conversion of 4-hydroxyphenylpyruvate to HGA. In the absence of HmgA, HGA accumulates and oxidizes to form HGA-melanin. We propose that the synthesis of turbomycin A and turbomycin B in *E. coli* carrying 4HPPD homologs results from a pathway that relies on both normal *E. coli* genes for indole production and the introduced 4HPPD family gene for HGA production (Fig 2). Concurrently, the majority of the HGA present spontaneously oxidizes and polymerizes to form the predominant melanin complex, which we predict serves as a catalyst in the formation of turbomycin products. This model is supported by our observation that addition of HGA to our cell-free system leads to an increased production of turbomycins. All of the turbomycin-producing clones were also hemolytic, which is likely due to the formation of HGA-melanin.

Many cultured bacteria, including *V. cholerae*, *Hyphomonas* sp., *S. colwelliana*, and *Yarrowia lipolytica*, produce HGA-melanin, resulting from the oxidation and polymerization of HGA (7, 24). HGA melanin accumulation also results from the expression of genes of the 4HPPD family from both eukaryotic and prokaryotic sources in *E. coli* (10, 23, 24, 30, 35, 36). 4HPPD proteins in eukaryotes tend to be significantly larger

than those of the prokaryotic family members, as reflected in human and *Arabidopsis* 4HPPD proteins, which are an estimated 50 and 45 kDa, respectively (30, 34). HGA production in plants is critical for the production of plastoquinone and tocopherols, essential quinine compounds, and growth of 4HPPD mutant lines can be rescued either by expression of 4HPPD or by application of exogenous HGA (29, 30). In humans, the role of 4HPPD can be more directly correlated to tyrosine degradation, since humans deficient in 4HPPD function are at risk for tyrosinemia type III, a disorder caused by the accumulation of tyrosine and phenolic metabolites that leads to mental retardation or neurological symptoms (12).

Other researchers, also using the metagenomic approach, identified a clone of unknown phylogenetic origin that results in the production of both an unidentified antimicrobial activity and a brown pigment in *E. coli*. A causal relationship between phenotypes and individual ORFs was not established, but we note that one of several ORFs present on the clone contains homology to 4HPPD (27). Given our observations, we expect that this single ORF is likely responsible for the observed phenotypes. Although the turbomycins in these other systems were not described, considering our recovery rate of metagenomic library clones producing increased levels of turbomycin molecules, we predict that turbomycin products are present but uncharacterized in many native environments that include indole production and were likely present in previously reported clones. These triaryl cations might be utilized by organisms in the soil as competition factors or for intercellular signaling.

This approach enabled us to identify molecules that were previously undetected in *E. coli* cultures. Although turbomycin synthesis requires only one introduced gene and therefore the function could have been discovered with libraries carrying smaller inserts, the large inserts reduced the number of clones that had to be screened to detect the activity. For example, the gigabase of DNA represented in our library would have required 250,000 clones in a vector that carried inserts of approximately 4 kb, thus necessitating a tremendous screening effort to obtain the same coverage.

The discovery of turbomycin production in *E. coli* suggests that metagenomics will be useful to access or create small molecules through enhancing the biosynthetic capacity of host cells. In particular, we have illustrated the possibility that creating chimeric pathways by introducing environmental DNA into host cells may provide access to small molecules that would not be discovered by traditional methods. Our results also suggest that expanding the host cell range for maintenance of heterologous sequences will, by altering the metabolic backgrounds in which metagenomic DNA is expressed, increase the likelihood of future discoveries of new natural products.

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