Palmitoylputrescine, an Antibiotic Isolated from the Heterologous Expression of DNA Extracted from Bromeliad Tank Water†

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Heterologous expression of large fragments of microbial DNA extracted directly from environmental samples (environmental DNA, or eDNA) in easily cultured hosts should provide access to some of the natural products produced by previously uncultured bacteria. The natural product antibiotic palmitoylputrescine (1) was isolated from Escherichia coli transformed with a cosmid (pCSLF16) containing DNA extracted directly from Costa Rican bromeliad tank water. In this report we describe the characterization of this antibiotic and its biosynthetic gene.

Bromeliad tanks, which trap organic matter and pools of water, are home to a rich assortment of both cultured prokaryotes and eukaryotes.1 Because they supply water to so many different organisms that live in the canopies of tropical and neotropical forests, tank bromeliads are considered a "keystone species" for maintaining biodiversity in these forests.2 With uncultured bacteria thought to outnumber their cultured counterparts by at least 2–3 orders of magnitude,3,4 bromeliad tanks are also likely to contain a rich assortment of yet uncultured microbes. Many factors contribute to our inability to culture the vast majority of bacteria that are present in environmental samples. For some microbes, growth in pure culture may require very specific culture conditions that have not yet been identified, while other bacteria may survive only in complex consortia and therefore, by definition, will never be obtained in pure culture.5,6 Whatever the reason, the inability to obtain these organisms in pure culture has meant that they have not been studied using traditional approaches to characterize natural products from bacteria.

The heterologous expression of DNA extracted directly from environmental samples (environmental DNA, eDNA) in easily cultured hosts should provide access to many of the natural products produced by uncultured bacteria. We have worked on an approach for accessing the natural products of uncultured microorganisms, especially soil microflora, by heterologously expressing eDNA in Escherichia coli-based cosmid libraries.7–11 In an attempt to expand this approach beyond soil, a cosmid library of DNA extracted directly from bromeliad tank water collected in Costa Rica was constructed in E. coli and screened for the expression of antibiotic activity (Figure 1). In this report we describe the characterization of a natural product antibiotic, palmitoylputrescine (1) (Figure 2), and its biosynthetic gene (Figure 3) that were isolated from an antibacterial active cosmid clone found in a Costa Rican bromeliad tank water eDNA library hosted in E. coli.

Results and Discussion

DNA extracted directly from bromeliad tank water collected in Costa Rica was blunt ended, ligated into the pWEB cosmid, and then transfected into E. coli using lambda phage. The resulting bromeliad tank water library was screened for clones that produce antibacterial activity using a top agar overlay assay containing Bacillus subtilis. CSLF16, an antibacterial active clone found in this primary B. subtilis overlay assay, was selected for more extensive characterization when it was found that the antibacterial activity produced by this clone could be extracted from liquid cultures into ethyl acetate and thus was likely a small organic molecule. Using B. subtilis as the test organism, a bioassay-guided fractionation of antibacterial active ethyl acetate extracts from cultures of CSLF16 led to the isolation of a single antibacterial active compound (1).

The structure of active compound 1 was elucidated by 1- and 2-D NMR and then confirmed by comparison with a synthetic sample (Figure 2). Deshielding of the carbons (Δ 40.5, 39.5) at each end of the four-carbon methylene spin system seen in 1H–1H COSY experiments suggested the presence of a 1,4-butanediamine (putrescine) substructure. Only two nitrogens are predicted to be present by HR-FABMS, and therefore the positive ninhydrin test requires that one of the nitrogen atoms of the putrescine be present as a primary amine. A long-range 1H–13C HMBC correlation from the only carbonyl (Δ 176.7) observed in the 13C spectrum to C4 of putrescine indicated that the second nitrogen predicted to be present by HR-FABMS must be present as an amide. The remaining atoms (C15H13) predicted by HR-FABMS to be present in the antibiotic compose a fully saturated 15-carbon aliphatic chain attached to the C-5 carbonyl and are seen as a methyl triplet (Δ 0.90), two methylene multiplets (Δ 2.18 and 1.62), and a large methylene envelope (Δ 1.2–1.4) in the 1H NMR spectra (Figure 1). The antibiotic produced by CSLF16 is therefore palmitoylputrescine (1). Synthetic monosubstituted palmitoylputrescine, which was prepared from putrescine and the N-hydroxysuccinimide ester of palmitic acid, was found to be spectroscopically identical to the natural sample.

To identify the genes responsible for the biosynthesis of palmitoylputrescine, the cosmid from CSLF16 pCSLF16 was transposon mutagenized and the DNA surrounding transposon insertions in cosmids that no longer conferred the production of antibiotic to E. coli was sequenced. Annotation of the eDNA sequence obtained from the transposon mutagenesis experiment indicated that a single 207 amino acid open reading frame (ORF), predicted to be a palmitoylputrescine synthase (Pps), was responsible for the observed antibacterial activity (Figure 3a). In a BLAST search against deposited sequences, the conceptually translated protein from this ORF showed no significant sequence identity (>20%) to any other deposited sequence. Transposon insertions in a region upstream of this ORF, which

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contains a sequence predicted to function as a prokaryotic promoter, also knock out production of antibiosis (Figure 3b). To confirm that the proposed Pps was necessary and sufficient to produce palmitoylputrescine, this ORF was subcloned in pGEX-3X as a GST fusion protein and retransformed into E. coli. When transformed into E. coli, the GST fusion construct was sufficient to confer the production of palmitoylputrescine to the host.

A 9.3 kb region of pCLSF16 that contains the proposed Pps and is flanked by two EcoNI restriction sites was subsequently sequenced and annotated (Figure 3a). In addition to the proposed Pps seven other large ORFs were found in this eDNA sequence. When the conceptually translated proteins from these ORFs were used in BLAST searches, only one of the predicted proteins showed significant identity (>20%) to any deposited sequences. This large ORF (shown shaded in Figure 3a, 766 amino acids) is related (40% identity) to a family of conserved hypothetical proteins of unknown function.

Although BLAST searches with the conceptual translation of Pps did not identify any closely related sequences, this ORF does show a weak relationship to a family of N-acetyltransferases (GCN5-related N-acetyltransferase, GNAT) in a Pfam search. The GNAT superfamily is composed of a diverse group of N-acetyltransferases, many of which have been shown to use acyl-coenzyme A (acyl-CoA) as cofactors for the acylation of primary amines. Bacterially derived long chain N-acyl derivatives of simple amines are often isolated as mixtures (2, 3, and 4). Two families of enzymes, autoinducer synthases (AISs) from proteobacteria and N-acyl amino acid syn-

Figure 1. Overview of the process used to construct and screen eDNA libraries for antibacterial active clones. For this study, bromeliad tank water collected in Costa Rica was used as a source of eDNA. High molecular weight eDNA is extracted directly from an environmental sample (a) and then concentrated by 2-propanol precipitation (b). The concentrated eDNA sample was then separated on a preparative agarose gel (c). Purified HMW eDNA was recovered by electroelution from the preparative gel and blunt ended (d). The blunt-ended eDNA was then ligated into a cosmid (e) and packaged into lambda phage (f). The library that resulted from transfecting the packaged cosmids into E. coli (h) was selected with kanamycin (i), allowed to mature for 2–4 days, and then overlaid with top agar containing B. subtilis (j). Clones producing zones of growth inhibition in the top agar were picked through the top agar and cleared of the assay strain by streaking on plates containing kanamycin and ampicillin (k).

Figure 2. Important spectral data used in elucidating the structure of palmitoylputrescine (1).

Figure 3. (a) Open reading frames found in a 9.3 kb EcoNI fragment of CSLF16 that contains the proposed palmitoylputrescine synthase. The positions of transposon insertions in CSLF16 that knock out the production of antibiosis are shown as open flags, and the proposed palmitoylputrescine synthase (Pps) is shown in black. Predicted ORFs that do not have any obvious homologues among deposited sequences are shown in white. The ORF shown in gray is related to a family of conserved proteins of unknown function. (b) A predicted prokaryotic promoter sequence upstream of the proposed palmitoylputrescine synthase start site.
Experimental Section

Library Construction. Five hundred milliliters of tank water was collected from Costa Rican bromeliads and passed through a fine sieve to remove large pieces of plant and animal material (Figure 1a). Bromeliad tank water was collected from epiphytes found in central Costa Rica at a coffee plantation that is now being returned to the wild by the Instituto Nacional de Biodiversidad (INBio finca). Fifty milliliters of 20% SDS, 50 mL of 1.0 M Tris pH 8.0, 40.5 g of NaCl, and 5 g of hexadecyltrimethylammonium bromide (CTAB) were added directly to 450 mL of the resulting tank water suspension, and the mixture was heated at 70 °C for 2 h. Tank water eDNA was precipitated from the centrifuge-clarified supernatant of this mixture using 0.6 volume of 2-propanol (Figure 1b). The precipitated eDNA was collected by centrifugation (10,000g/15 min), washed with 70% ethanol, and resuspended in Tris-EDTA pH 7.6 (6 mL) (Figure 1b). The crude tank water extract was separated overnight on a preparative 1.0% agarose gel (1 h at 100 V and then overnight at 20 V), at which point the edges of the gel were removed and stained with ethidium bromide. The band of high molecular weight (HMW) eDNA that separated from other environmental contaminants and smaller sheared eDNA was electroeluted (100 V, 3 x for 45 min) from a slice of the remaining unsheared preparative gel (Figure 1c). Purified HMW eDNA was blunted ended (EndIt, Epicentre) (Figure 1d), ligated into precut dephosphorylated pWEB cosmids (Figure 1e), packaged into lambda phage (Figure 1f), and then transfected into E. coli (EPI-100) (Figure 1g).

Antibacterial Screening. Bromeliad tank water clones were selected on Luria-Bertani (LB) agar plates containing kanamycin (Figure 1i) and screened for the production of antibacterial activity using a top agar overlay assay (Figure 1j). Cosmid clones initially selected on LB plates containing kanamycin (Figure 1i) were allowed to incubate at 30 °C for 24 h and then after an additional 2–4 days at room temperature, the mature colonies were overlayed with top agar containing kanamycin-resistant B. subtilis (Figure 1j). Colonies that produced zones of growth inhibition in the B. subtilis lawn, indicating the production of antibacterial activity, were picked through the top agar and cleared of the ampicillin-sensitive assay strain by streaking onto LB plates containing ampicillin (Figure 1k). Active colonies were then grown in liquid shake culture and assayed for the production of ethyl acetate extractable and bacterial active antibacterial activity.

Culture Conditions, Isolation, Characterization, and Synthesis. Ethyl acetate extracts from cultures of the antibacterial active cosmids clone designated CSLF16 were active against B. subtilis and produced a bioautography pattern not previously seen in assays run on extracts from soil eDNA clones. In bioautography experiments dried TLC plates containing crude extracts (silica gel 90:10 CHCl3–MeOH) were overlayed onto LB agar plates containing B. subtilis for 1–2 h. The agar plates were then incubated overnight at 30 °C, and the patterns of growth inhibition in the bacterial lawns that developed were recorded. A bioassay-guided fractionation of the antibacterial active ethyl acetate extract from CSLF16 was undertaken using B. subtilis (Figure 1l). Extracts from large-scale cultures of CSLF16 grown in LB for 3 days at 30 °C were partitioned by normal-phase flash chromatography using a CHCl3–MeOH step gradient. Antibacterial activity that eluted from the normal-phase column with 30% methanol was then further partitioned by reversed-phase flash chromatography (CH3OH–H2O modified with 0.1% CF3COOH). The antibacterial activity eluted from the reversed-phase column with 70% methanol and stained with ninhydrin, indicating the presence of a primary amine. Synthetic palmitoylputrescine was prepared by stirring a 100-fold excess of putrescine with the N-hydroxysuccinimide ester of palmitic acid (50 mg) in 1 mL of a 1:1 mixture of ethyl acetate and THF for 18 h at 24 °C. The completed reaction was extracted three times with ethyl acetate, and palmitoylputrescine was purified (90% yield) from the crude extract by reverse-phase (C18) flash chromatography (CH3OH–H2O step gradient).

Palmitoylputrescine (1): white powder; H NMR (500 MHz, CD3OD) 3.20 (C4, 2H, t, 7), 2.94 (C1, 2H, t, 7.5), 2.18 (C6, 2H, t, 8), 1.65 (C2, 2H, m), 1.64–1.56 (C3, C7, 4H, m), 1.2–1.4 (m), 0.90 (C20, 3H, t, 7); 13C NMR (100 MHz, CD3OD) 176.7 (C5), 40.5 (C1), 39.5 (C4), 37.3 (C6), 33.2, 30.9 (m), 30.8, 30.6, 30.5, 27.7 (C3), 27.2, 26.0 (C2), 23.9, 14.6 (C20); HR-FABMS m/z 327.3375 [M]+ (calcd for C15H27NO2, 327.3375).

Pps Sequencing and Cloning. pCSLF16, the cosmids isolated from CSLF16, was randomly transposon mutagenized with donor plasmid pGPS2.1 using the Genome Primary System (GPS, NEB). E. coli transformed with the mutagenized
Cosmids was assayed for antibacterial activity in the same overlay assay used to screen the primary library. Cosmids were isolated from inactive colonies, and the eDNA sequence surrounding each of the transposon insertions was determined using primers S and N (NEB) that are specific for the GPS surrounding each of the transposon insertions was determined.

The sequence derived from the knockouts was isolated from inactive colonies, and the eDNA sequence used to construct the eDNA sequence responsible for the transposon. The sequence derived from the knockouts was observed antibacterial activity. All of the DNA analysis was used to construct the eDNA sequence responsible for the transposon. The sequence derived from the knockouts was isolated from inactive colonies, and the eDNA sequence used to construct the eDNA sequence responsible for the transposon.

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Supporting Information Available: 13C NMR and 1H NMR spectra for palmitoylputrescine (1). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes


