Cultured soil bacteria have been an important source of biologically active, naturally occurring small molecules. Uncultured soil bacteria, which outnumber their cultured counterparts by at least two orders of magnitude, are likely to be an equally important source of such molecules. In an attempt to access the biosynthetic potential of uncultured soil bacteria, we have explored an approach that involves the cloning and heterologous expression of DNA extracted directly from environmental samples (environmental DNA, eDNA) into readily cultured bacteria. Earlier, we described the identification and characterization of small-molecule antibiotics from antibacterially active eDNA clones found using a high-throughput phenotypic screen.[5] This approach directly couples the biosynthesis of each natural product that is found to be a relatively small piece of cloned eDNA and therefore permits the characterization of both new natural products and their biosynthetic gene clusters simultaneously. Herein, we report the isolation and characterization of the isocyanide-containing eDNA-derived antibiotic 1, its biosynthetic genes (isnA and isnB), the origin of the isocyanide nitrogen atom, and the general outline of the biosynthesis of 1.

CSLG18, the eDNA clone that produces 1, was found in a cosmid library constructed from blunt-ended gel-purified high-molecular-weight eDNA extracted directly from soil collected in Boston (MA, USA). The cosmid library was screened for antibacterially active clones by using a top agar overlay which contained Bacillus subtilis.[6] Clones that produced a zone of growth inhibition in the overlaid B. subtilis lawn were recovered from the assay plates and tested for the production of extractable organic antibacterial activity.[1]

Bioassay-guided fractionation of the antibacterially active extract in ethyl acetate from cultures of CSLG18 led to the isolation of 1, an isocyanide-functionalized C3-substituted indole. The indole and trans olefin were readily inferred from standard 1D and 2D NMR spectroscopic experiments.[2] The triplet resonance observed at $\Delta \delta = 107.4$ ppm in the $^{13}$C NMR spectrum suggested the presence of the isocyanide functionality as $^{13}$C–$^{14}$N coupling between the nitrogen atom and the adjacent carbon atom of the isocyanide group results in a triplet resonance for the carbon atoms, which is a hallmark of this functional group. The proposed structure of 1 was subsequently confirmed by single-crystal X-ray diffraction (Figure 1).[3] Although the cis isomer of 1 has been obtained from cultured bacteria, to the best of our knowledge 1 has never been reported as a natural product.[4]

The genes responsible for the biosynthesis of 1 were sought in pCSLG18.1, a 5.5-kb antibacterially active subclone of the eDNA cosmid, pCSLG18.[5] Two predicted open-reading frames (ORFs) contained in pCSLG18.1 (Figure 1) were identified by transposon mutagenesis as necessary for the production of 1 and have been given the names isnA and isnB (isocyanide, isonitrile), respectively. The predicted translation product of isnA shows the highest-sequence identity to predicted proteins related to PveA of pyoverdine chromophore biosynthesis (33%) and Dit1 of yeast-spor wall biosynthesis (21%). Neither PveA nor Dit1 has been functionally characterized, although both are thought to be involved in the biosynthesis of C–N bonds.[5,7] The predicted translation product of isnB shows the highest-sequence identity to non-heme iron α-ketoglutarate dependent oxygenases, including the oxygenase from clavaminate biosynthesis and PvcB, a second enzyme thought to be involved in pyoverdine chromophore biosynthesis.[8] Three additional ORFs (>100 amino acids) were found in CSLG18.1; ORFs 4 and 5 are related to hypothetical proteins of unknown function and ORF 3 is related to prenyltransferases.

The successful expression of isnA and isnB (isnA/B), the biosynthetic genes for 1, in E. coli allowed the biosynthesis of 1.
producing organisms. To assess the role of tryptophan in the biosynthesis of 1, a wild-type (transaminase-proficient) strain of E. coli (strain1) transformed with isNA/B was used to elucidate the overall scheme for the biosynthesis of 1 (Table 1). Cultures of E. coli that overexpressed either IsnA-GST or IsnB-GST individually did not accumulate 1. However, 1 accumulated in the culture broth when these two strains were co-cultured in the same flask. IsnA and IsnB are therefore necessary and sufficient to confer the production of 1 to E. coli, and one of the two enzymes must produce a diffusible intermediate that is converted into 1 by the other. E. coli that express IsnA-GST grown in media preconditioned with the growth of E. coli that express IsnB-GST do not accumulate 1 in the culture broth. The diffusible intermediate is therefore produced by IsnA and converted into 1 by IsnB (Scheme 1). All attempts to isolate this intermediate from cultures that either overexpress IsnA-GST or are transformed with isNA/B were unsuccessful.

N-formyl and N-methyl compounds have been proposed as possible isocyanide intermediates in what could be a two- or three-step isocyanide biosynthetic scheme. However, N-formyl and N-methyl derivatives of tryptophan and tryptamine (0.1 mg/mL) did not serve as substrates for the

Table 1: Results from feeding experiments used to decipher the overall biosynthetic approach to 1.

<table>
<thead>
<tr>
<th>E. coli (strain 1)</th>
<th>E. coli (strain 2)</th>
<th>Culturing treatment</th>
<th>(^{13}N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>isNA+isNB</td>
<td>isNA-GST</td>
<td>cocultured</td>
<td>+</td>
</tr>
<tr>
<td>isNA-GST</td>
<td>isNB-GST</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>isNA-GST + isNB-GST</td>
<td>isNA-GST</td>
<td>preconditioned</td>
<td>+</td>
</tr>
<tr>
<td>isNB-GST</td>
<td>isNA-GST</td>
<td>preconditioned</td>
<td>-</td>
</tr>
<tr>
<td>isNB-GST</td>
<td>N-methyl tryptophan</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>isNB-GST</td>
<td>N-formyl tryptophan</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>isNB-GST</td>
<td>N-methyl tryptamine</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>isNB-GST</td>
<td>N-formyl tryptamine</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. The m/z values observed for 1 obtained from cultures of E. coli transformed with isNA/B and grown in either \(^{14}N\)-tryptophan or \(^{15}N\)-tryptophan. A transaminase-deficient (aspC, ilvE, and tyrB) E. coli strain was used in these feeding experiments to prevent the loss of the labeled amine group.

Scheme 1. Feeding experiments in E. coli indicate IsnA uses tryptophan in the biosynthesis of 1. The proposed intermediate 2 is suggested by feeding experiments that use the methyl ester 3.
production of 1 when added to cultures that overexpress IsnB-GST. It seemed plausible that IsnA might form the isocyanide function in a single enzymatic step and that the role of IsnB would be the oxidative decarboxylation of the proposed isocyanide-functionalized intermediate 2. In our hands, the synthetic intermediate 2 was not stable and, therefore, could not be used in feeding experiments to test this hypothesis. However, we were able to synthesize the methyl ester of 2 (3) and thought that the slow cleavage of the methyl ester by promiscuous bacterial esterases might circumvent the instability problem.[12] The addition of 3 (0.1 mgmL−1) to cultures that express IsnB-GST led to the accumulation of 1 in the culture broth, and when the cultures of CSLG18.1 were spiked with 3, they showed increased production of 1 relative to the control cultures of CSLG18.1. Compound 1 was not detected in the extracts in ethyl acetate from the vector control cultures spiked with 3 or from IsnA-GST cultures spiked with 3. These feeding studies suggest that the isocyanide group seen in 1 is formed in a single enzymatic step on IsnA and that the proposed isocyanide-containing intermediate 2 is oxidatively decarboxylated by IsnB to give 1 (Scheme 1). The lack of stability of 2 is likely to be responsible for our inability to isolate the proposed intermediate.

The eDNA approach, used in this study to discover antibiotic 1, directly couples natural products with their biosynthetic gene clusters, thus providing both a means for the isolation of previously inaccessible natural products from uncultured bacteria and a direct link to their biosynthetic enzymes. Characterization of the antibacterially active eDNA clone CSLG18 led to the identification of the isocyanide-containing natural-product antibiotic 1, and in turn to the first isocyanide synthase, IsnA. The origin of the isocyanide carbon atom is an intriguing, but unanswered question, for all naturally occurring isocyanides. This question will be addressed in due course.

Keywords: biosynthesis · DNA · heterologous expression · isocyanides · natural products


[2] 13CNMR (125 MHz, CD3Cl): δ = 163.9, 137.5, 130.7, 127.3, 125.1, 123.8, 121.8, 120.3, 111.24, 107.4 ppm (t); 1H NMR (500 MHz, CD3OD): δ = 8.58 (1H, s), 7.68 (1H, d, 7.5), 7.45 (1H, d, 8), 7.39 (1H, d, 3), 7.28 (1H, d, 14.5), 6.37 ppm (1H, d, 14); HRMS (ESI-TOF): m/z calcd for C13H14N3 · H2O = 239.11; found 239.11 [M + H]+; see the Supporting Information for a description of the isolation procedure, structure-elucidation methods, and the 1H and 13CNMR spectra of 1.

[3] CCDC-273844 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.


[10] D. M. LeMaster, F. M. Richards, Biochemistry 1988, 27, 142; see the Supporting Information for a detailed description of the methods used in these feeding experiments.
