J. Microbiol. Biotechnol. (2013), **23**(6), 850–855
http://dx.doi.org/10.4014/jmb.1212.12008
First published online March 21, 2013
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Bacillus subtilis as a Tool for Screening Soil Metagenomic Libraries for Antimicrobial Activities

Biver, Sophie*, Sébastien Steels, Daniel Portetelle, and Micheline Vandenbol

Microbiology and Genomics Unit, Gembloux Agro-Bio Tech, University of Liège, B-5030 Gembloux, Belgium

Received: December 4, 2012 / Revised: January 17, 2013 / Accepted: February 2, 2013

Finding new antimicrobial activities by functional metagenomics has been shown to depend on the heterologous host used to express the foreign DNA. Therefore, efforts are devoted to developing new tools for constructing metagenomic libraries in shuttle vectors replicatable in phylogenetically distinct hosts. Here we evaluated the use of the *Escherichia coli–Bacillus subtilis* shuttle vector pHT01 to construct a forest-soil metagenomic library. This library was screened in both hosts for antimicrobial activities against four opportunistic bacteria: *Proteus vulgaris*, *Bacillus cereus*, *Staphylococcus epidermidis,* and *Micrococcus luteus*. A new antibacterial activity against *B. cereus* was found upon screening in *B. subtilis*. The new antimicrobial agent, sensitive to proteinase K, was not active when the corresponding DNA fragment was expressed in *E. coli*. Our results validate the use of pHT01 as a shuttle vector and *B. subtilis* as a host to isolate new activities by functional metagenomics.

Key words: Antimicrobial activity, *Bacillus cereus*, *Bacillus subtilis*, functional metagenomics, shuttle vector

Soils contain a high diversity of microorganisms expressing various enzymes and antimicrobial agents potentially useful in agriculture, the chemical industry, and both human and veterinary medicine [9]. As most of these organisms (>99%) are unculturable by standard laboratory techniques [27, 28], methods have been developed to access this reservoir of natural products by means of nucleic acid analysis [10]. Total DNA from an environmental sample is extracted and new genes or activities are sought by DNA sequence analysis or function-based screening. This last approach, called functional metagenomics, allows the discovery of completely new types of enzymes showing only low similarity to known proteins and, thus, whose functions cannot be deduced from their sole sequences. The method consists of cloning the environmental DNA in an easily cultivable host, typically *Escherichia coli*, and screening the metagenomic libraries obtained for the desired activities. Screening is done on medium supplemented with an enzyme substrate or with an indicator organism against which an antimicrobial activity is sought. This strategy has been applied to several ecosystems, allowing the detection of a few antibiotics [6, 14, 17, 18] and diverse enzymes such as cellulases, xylanases, glucosidases, esterases, amylases, and proteases [11, 16, 20, 24, 26].

The main limitation of functional metagenomics in new molecule discovery is its reliance on the ability of the surrogate host to recognize the foreign transcription and translation start sequences (promoters and ribosome-binding sites) and provide the factors needed for correct assembly and targeting of the heterologous polypeptides (protein-modifying enzymes, chaperones, cofactors, proper secretion machinery, *etc*.) [13]. To favour expression of genes of different phylogenetic origins, it is therefore advantageous to screen the libraries in various host bacteria. Yet to date, only a few metagenomic studies have used a host other than *E. coli* to isolate new molecules. Shuttle vectors have been designed and successfully used to express metagenomic libraries in several Gram-negative proteobacteria [7, 8, 22, 31] and in the high-GC Gram-positive strain *Streptomyces lividans* [19, 30]. In most cases, the new activities were not observable when the genes were cloned in *E. coli*, which confirms the need to screen libraries in different host bacteria.

Here we report the analysis of a forest-soil metagenomic library using the low-GC Gram-positive *B. subtilis* as a host to find new interesting activities. The library was constructed in the *E. coli–B. subtilis* shuttle vector pHT01 (MoBiTec) and screened in both hosts for antimicrobial activities against four opportunistic bacteria.

*Corresponding author
Phone: +32-81622355; Fax: +32-81611555;
E-mail: Sophie.Biver@ulg.ac.be

Materials and Methods

Bacterial Strains

The libraries were constructed in ElectroMAX DH10B *Escherichia coli* (Invitrogen) and in *Bacillus subtilis* strain 1012 (MoBiTec). The indicator strains used to test for antimicrobial activities were *Proteus vulgaris* (laboratory collection), *Bacillus cereus* ATCC 10876, *Staphylococcus epidermidis* ATCC 12228, and *Micrococcus luteus* LMG 3293. All subcloning steps were performed in Subcloning Efficiency DH5α Chemically Competent *E. coli* (Invitrogen).

Library Construction in *E. coli*

A plasmid library was constructed with metagenomic DNA isolated from a soil sample taken from the A horizon of a Belgian deciduous forest. The sample was collected from the upper 5 cm layer below the litter in Groenendaal at the end of October 2010. The library was constructed in the *E. coli*–*B. subtilis* shuttle vector pHT01 (MoBiTec) [2]. Briefly, metagenomic DNA was partially digested with *Sau*3AI, and fragments ranging in size from 9 to 20 kb were ligated into the *Bam*HI-linearized, dephosphorylated vector. The ligation products were introduced into electrocompetent *E. coli*. This yielded a library with an average insert size of 12 kb. The colonies were pooled in 2×YT medium containing 100 μg/ml ampicillin and incubated at 37°C for 60 min. The cells were then harvested and their plasmids extracted.

Bacillus subtilis Transformation and Library Construction

Plasmid DNA from the *E. coli* library was used to transform electrocompetent *B. subtilis* cells. The cells were prepared according to the protocol of Cao *et al.* [3], with several modifications. An overnight culture of *B. subtilis* (grown in LB) was diluted 50 times (OD_{600nm} ~ 0.02) in 50 ml of LB containing 0.5 M sorbitol. The culture was incubated at 37°C under shaking until the OD_{600nm} reached 0.7. The cells were centrifuged at 5,000 ×*g* for 6 min (4°C) and washed three times with 10 ml of an ice-cold solution containing 0.5 M sorbitol, 0.5 M mannitol, and 10% (v/v) glycerol. They were washed once more with 1 ml of electroporation medium [0.5 M trehalose, 0.5 M sorbitol, 0.5 M mannitol, 10% (v/v) glycerol] before resuspension in 500 μl of the same medium. For electroporation, the competent cells were aliquoted in 1.5 ml tubes (60 μl/tube) and incubated for 5 min on ice with 2 μl dialyzed DNA (800 ng). They were then transferred to ice-cold electroporation cuvettes (1 mm electrode gap) and electroporated (25 μF, 200 Ω, 2.2 kV). After a 2 h incubation at 37°C (without shaking) in 1 ml of recovery medium (LB medium with 0.5 M sorbitol and 0.38 M mannitol), the cells were spread on LB plates (+ 5 μg/ml chloramphenicol for plasmid selection) and incubated overnight at 37°C.

The average insert size of the library was determined by analyzing 30 randomly selected clones. Their plasmids were isolated from 15 ml overnight cultures (LB + 30 μg/ml chloramphenicol) using the High Pure Plasmid Isolation Kit (Roche). To improve bacterial lysis, 5 mg/ml lysozyme was added to the Suspension Buffer and the cells were incubated at 30°C for 30 min. The isolated plasmids were digested with *Bam*HI and *Hin*dIII to estimate the size of the DNA inserts.

Library Screening for Antimicrobial Activities in *E. coli*

Each colony was collected into a well of a 96-well plate containing 2×YT medium with 50 μg/ml ampicillin and incubated at 37°C for 16 h before replication and long-term storage at −80°C. To screen for antimicrobial activities against *P. vulgaris* and *B. cereus*, the colonies were replicated with a 96-pin replicator on 2×YT agar (+ 50 μg/ml ampicillin) and allowed to grow for one night at 37°C and then for 2–3 days at room temperature. A lawn of *P. vulgaris* or *B. cereus* (in 2×YT–agar 0.7% with ampicillin) was poured onto the *E. coli* plates, which were next incubated at 29°C for 16 h. Activities against *S. epidermidis* and *M. luteus* were sought by replicating the transformants directly on freshly poured lawns of indicator bacteria (2×YT–agar 1.5% without antibiotics). Plates were incubated at 37°C for 16 h.

Library Screening for Antimicrobial Activities in *B. subtilis*

Transformants were transferred to 96-well microplates containing 2×YT medium with 30 μg/ml chloramphenicol and incubated at 37°C for 16 h before replication and long-term storage. Activities against *P. vulgaris*, *B. cereus*, and *M. luteus* were sought by replicating the transformants on freshly poured lawns of indicator bacteria (2×YT–agar 1% without antibiotics). Plates were incubated at 29°C (*P. vulgaris* and *B. cereus*) or 37°C (*M. luteus*) for 16 h.

DNA Sequencing, Sequence Analysis, and Subcloning

The plasmid from the positive clone pHT01-AMPcd4 was sequenced at GATC Biotech (Germany). Sequence similarity searches were carried out with the BLASTX and BLASTP programs. Bacterial operons were predicted with the FgenesB program (SoftBerry). Conserved protein domains and lipoprotein signal peptides were detected, respectively, with InterProScan [1] and the LipoP 1.0 server [15]. To determine which ORFs were responsible for the observed antimicrobial activity, the genes were subcloned individually or in small groups into the pHT01 vector (Fig. 2A). A *Sna*BI-*Ear*I fragment (5.7 kb) containing ORF 1 was subcloned in the vector opened with *Sna*BI and *Sma*I. The sticky ends generated by *Ear*I were blunt-ended with T4 DNA polymerase (New England Biolabs). ORFs 2–4 (3.2 kb) were recovered by *Pvu*II restriction and inserted

Table 1. Primers used for subcloning genes of the AMPcd4 insert.

ORF	Sequence of the forward primer (5'–3')	Sequence of the reverse primer (5'–3')
1 (partial)	cgcGGATCCTAAGATGAGGCGAGTTAACAGTCG	gcTCTAGACCATGGACGCGTGACGTG
2	cgcGGATCCGTATACAATCATAATGGCAGCAACG	gcTCTAGACTTCATAGATTATTAGGTCAGGATGGAAG
3	cgcGGATCCCAGGACGTAAATCAAAGTACTATAGAGGC	gcTCTAGAGATGAAAAGACAGATAAACGCCAGC
4	cgcGGATCCCGCCGATTAGGTTTTGGAATTG	gcTCTAGAAGGTTTCATAAACTAGAATTTGGGCG
5 (partial)	cgcGGATCCTGGCTGCGCAAAAATG	gcTCTAGACGCATAGCTTACAGAATCCAAACAC

Restriction sites are underlined.

into the *Sma*I site of pHT01. An *Hpa*I-*Sma*I fragment (1.4 kb) containing ORFs 5–6 was also cloned into the *Sma*I site of pHT01. The ORFs of the *Pvu*II subclone were amplified separately with primers coupled to the *Bam*HI or *Xba*I sites (Table 1) and introduced into the vector digested with these two enzymes. All constructs were checked by DNA sequencing.

Analysis of Cell Culture Supernatants

Overnight cultures of transformed *B. subtilis* were diluted 50× in 2×YT medium supplemented with 30 µg/ml chloramphenicol and incubated at 37°C for 3 h before addition of 0 or 10 µM IPTG. After incubation for three more hours (OD_{600nm} ~ 1), the supernatants were collected by centrifugation for 10 min at 10,000 ×*g* (4°C) and then filtered through a 0.22 µm filter. To test their antibacterial activity, the supernatants (35 µl) were deposited in wells made in a freshly seeded lawn of *B. cereus* (in 2×YT–agar 1.5%) (agar well diffusion method). The plates were incubated at 29°C for 16 h. The proteinaceous nature of the observed antimicrobial activity was checked by pre-incubating 500 µl of each cell-free supernatant with 5 µl of a 20 mg/ml solution of proteinase K at 37°C for 1 h before adding the supernatants to the agar wells.

Nucleotide Sequence Accession Numbers

The nucleotide sequence corresponding to the pHT01-AMPcd4 candidate was deposited in the GenBank database under the accession number KC238307.

RESULTS AND DISCUSSION

Construction of Metagenomic Libraries

Environmental DNA from a forest soil was introduced into shuttle vector pHT01 (8 kb; MoBiTec), which can replicate in both *E. coli* and *B. subtilis*. A library was first constructed in *E. coli* with DNA fragments 9–20 kb long (average insert size: 12 kb) [2]. About 99% of the 35,000 colonies composing this *E. coli* library contained inserts 9–20 kb in size. Their plasmids were isolated and used to transform electrocompetent *B. subtilis* cells. Like most Gram-positive bacteria, *B. subtilis* was hard to transform, especially with the large plasmids composing the library (up to 28 kb). Bacterial transformation was possible only when the electrocompetent bacteria were prepared from cells harvested at an OD_{600nm} close to 0.7. As the transformation efficiency (~150 transformants/µg DNA for the library) was strongly dependent on plasmid size, the *B. subtilis* library contained only 50% inserts larger than 9 kb. Despite this size bias as compared with the same library hosted in *E. coli*, metagenomic fragments up to at least 18 kb were found in the *B. subtilis* library. This fragment size should be high enough to isolate antimicrobial activities encoded by small to medium-size operons but will not be sufficient for the recovery of very large gene clusters, such as those encoding modular polyketide synthases that may exceed 50 kb [4, 5].

Screening for Antimicrobial Activities in *E. coli* and *B. subtilis*

Antimicrobial assays were designed to screen the *E. coli* and *B. subtilis* libraries for antibacterial activities against four easily cultivable opportunistic pathogens: *Proteus vulgaris*, *B. cereus*, *S. epidermidis*, and *M. luteus*. Antibiosis tests are generally based on the top-agar overlay assay in which potential producers of antimicrobial agents (here, the transformants) are grown for a few days before pouring a lawn of indicator bacteria (within soft agar) onto the colonies. Antibacterial activities are then visualized by growth inhibition of the indicator bacteria around the colonies. This method is relatively sensitive, as the antimicrobial agents have time to accumulate around the colonies before the indicator bacteria are plated. However, it cannot be used for *B. subtilis* cells, as they form large colonies too easily dispersed by top agar overlays. Moreover, this method is hard to apply in library screening because the indicator strains must be resistant to the antibiotics required to maintain the plasmids during extended growth of the host bacteria (ampicillin in *E. coli* and chloramphenicol in *B. subtilis*). Only *P. vulgaris* and *B. cereus* are ampicillin-resistant, and all four indicator strains are sensitive to chloramphenicol. To circumvent these difficulties, we spotted

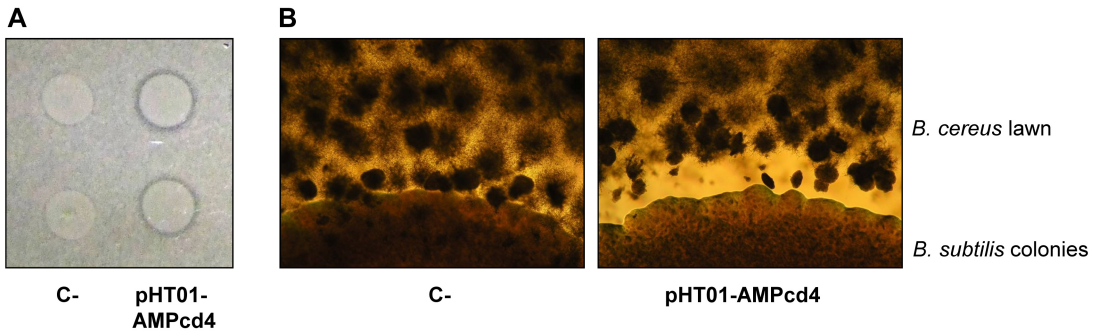

Fig. 1. Antibiosis test showing the antibacterial activity of the metagenomic clone pHT01-AMPcd4.
(**A**) Growth inhibition of the *B. cereus* lawn around *B. subtilis* colonies expressing the metagenomic fragment AMPcd4. C-, *B. subtilis* cells transformed with the empty vector pHT01. (**B**) Highest magnification of **A**.

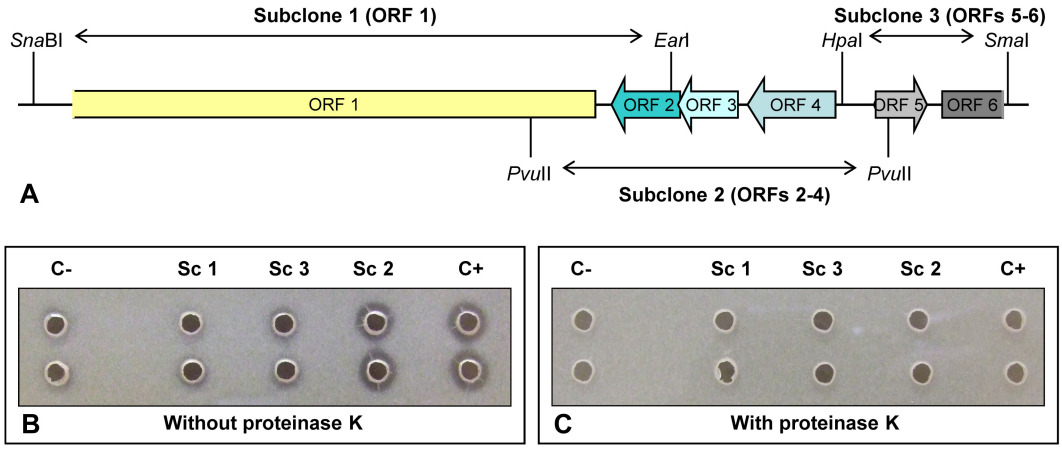

Fig. 2. Identification of the ORFs responsible for the antibacterial activity encoded by the AMPcd4 insert.
The insert responsible for the antibacterial activity was digested with several restriction enzymes, and three of the fragments obtained were subcloned in the pHT01 vector. *B. subtilis* cells transformed with each construct were tested for antimicrobial activity. (**A**) Schematic representation of the AMPcd4 insert and of the DNA fragments subcloned in the pHT01 vector. The three fragments were obtained by enzymatic restriction with *Sna*BI and *Ear*I (ORF 1), *Pvu*II (ORFs 2–4), or *Hpa*I and *Sma*I (ORFs 5–6). (**B**) Antimicrobial activity of *B. subtilis* expressing fragments of the AMPcd4 insert. Cell-free culture supernatants of *B. subtilis* transformed with the pHT01 vector (C-), the pHT01-AMPcd4 plasmid (C+), or one of the three constructs (Sc 1, Sc 2, or Sc 3) were tested for antimicrobial activity against *Bacillus cereus* by the agar well diffusion method. Antibacterial activity is indicated by a clear zone around the sample well. (**C**) Confirmation that a protein is responsible for the antibacterial activity. Culture supernatant from each *B. subtilis* strain was pre-incubated at 37°C for 1 h with 0.2 mg/ml proteinase K and tested for antimicrobial activity.

the transformants directly onto freshly seeded lawns of indicator bacteria without antibiotics (spot-on-lawn method) and incubated the plates for one night only to allow the transformants and the indicator strain to grow.

We screened about 13,000 *E. coli* colonies (~156 Mb), using either the top-agar overlay assay (*P. vulgaris* and *B. cereus*) or the spot-on-lawn method (*S. epidermidis* and *M. luteus*). The latter method was also used to screen the *B. subtilis* library (12,000 colonies, ~72 Mb) for activities against *P. vulgaris*, *B. cereus*, and *M. luteus* but not against *S. epidermidis*, which is naturally inhibited by *B. subtilis*. We did not find any activities with the *E. coli* library, but one antimicrobial activity against *B. cereus* was observed when the library was expressed in *B. subtilis* (Fig. 1). No activity was detectable when the pHT01-AMPcd4 plasmid responsible for inhibition of *B. cereus* growth was introduced into *E. coli* (data not shown). This validates *B. subtilis* as a heterologous expression host useful for detecting new antimicrobial agents from environmental DNA.

Analysis of the Positive Clone

DNA sequence analysis of the 8,435 bp insert of the positive clone did not reveal any similarity to known antimicrobial genes (Table 2). To find which ORFs were responsible for the observed activity, we subcloned the 6 putative genes of the insert individually (gene 1, subclone 1) or in groups (genes 2–4, subclone 2 and genes 5–6, subclone 3) downstream from the IPTG-inducible *Pgrac* promoter of the pHT01 vector (Fig. 2A), transformed *B. subtilis* with each construct, and tested culture supernatants of the subclones, grown with or without 10 μM IPTG, for their ability to inhibit *B. cereus* growth. Fig. 2B shows that at least one gene contained in the 3.2 kb insert of subclone 2 was required to induce an inhibition halo in the indicator

Table 2. BlastP results for the putative ORFs of the AMPcd4 insert.

ORF	Amino acids	Best hit [organism] accession number	Max identity	E-value
1 (partial)	1,580	Hypothetical protein Solca_4193 [*Solitalea canadensis* DSM 3403] YP_006258359	38%	2e-41
2	207	Hypothetical protein PMI10_03720 [*Flavobacterium* sp. CF136] ZP_10731837	26%	1e-12
3	180	Outer membrane protein [*Solitalea canadensis* DSM 3403] YP_006258357	46%	6e-39
4	265	Hypothetical protein A33Q_08172 [*Indibacter alkaliphilus* LW1] ZP_11014283	40%	4e-53
5	154	Xanthine guanine phosphoribosyl transferase [*Helicobacter bizzozeronii* CIII-1] YP_004608237	31%	0.36
6 (partial)	182	Hypothetical protein Ajs_2459 [*Acidovorax* sp. JS42] YP_986696	58%	2e-66

lawn. Similar results were obtained in the presence of IPTG (data not shown). The 3.2 kb fragment carries an ORF coding for a protein showing similarity to outer membrane proteins of the OmpH family and two ORFs encoding hypothetical proteins, one of which was predicted to possess a lipoprotein signal peptide (ORF 2). Interestingly, no growth inhibition was observed when these genes were expressed separately (data not shown), which suggests their organization in an operon, as also predicted by sequence analysis.

Microorganisms produce a wide variety of antimicrobial compounds, including polyketides, nonribosomal and ribosomal peptides, and also smaller molecules such as lactic acid, ethanol, and hydrogen peroxide [12, 21, 25, 29]. To gain more information about the nature of the new antimicrobial agent, cell-free supernatants were pre-incubated with 0.2 mg/ml proteinase K for one hour before testing their antibacterial activity by the well diffusion method. After treatment with the protease, no growth inhibition was observed. This suggests that a protein is responsible for the antimicrobial activity (Fig. 2C). Further analysis will be necessary to determine precisely the nature and activity spectrum of the new antibacterial agent, which might be relatively narrow as no activity was observed against *P. vulgaris* (Proteobacteria) and *M. luteus* (Actinobacteria). It will thus be interesting to test its activity against various species of the Firmicutes group and notably several *Bacillus* strains. Some antibacterial peptides have indeed been shown to have an extremely narrow activity spectrum, sometimes against only a few closely related bacterial strains [23].

In this study, we have evaluated the use of *Bacillus subtilis* as a host for screening metagenomic libraries for antibacterial activities. The strain proved hard to transform with large plasmids, but seems to maintain them stably, even after 16 h without antibiotics. This enabled us to find a new antibacterial activity against *B. cereus*, using the less usual spot-on-lawn method. This new activity could not have been isolated by screening in *E. coli*, which confirms the advantage of constructing metagenomic libraries in vectors, such as the *E. coli–B. subtilis* shuttle vector pHT01, that are replicatable in distinct host bacteria.

Acknowledgments

Sophie Biver is a Postdoctoral Researcher of the Fonds National de la Recherche Scientifique (F.R.S-FNRS).

REFERENCES

1. Apweiler, R., T. K. Attwood, A. Bairoch, A. Bateman, E. Birney, M. Biswas, *et al*. 2001. The InterPro database, an integrated documentation resource for protein families, domains and functional sites. *Nucleic Acids Res.* **29:** 37–40.
2. Biver, S. and M. Vandenbol. 2013. Characterization of three new carboxylic ester hydrolases isolated by functional screening of a forest soil metagenomic library. *J. Ind. Microbiol. Biotechnol.* **40:** 191–200.
3. Cao, G., X. Zhang, L. Zhong, and Z. Lu. 2011. A modified electro-transformation method for *Bacillus subtilis* and its application in the production of antimicrobial lipopeptides. *Biotechnol. Lett.* **33:** 1047–1051.
4. Chen, X. H., J. Vater, J. Piel, P. Franke, R. Scholz, K. Schneider, *et al*. 2006. Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB 42. *J. Bacteriol.* **188:** 4024–4036.
5. Chung, E. J., H. K. Lim, J. C. Kim, G. J. Choi, E. J. Park, M. H. Lee, *et al*. 2008. Forest soil metagenome gene cluster involved in antifungal activity expression in *Escherichia coli*. *Appl. Environ. Microbiol.* **74:** 723–730.
6. Courtois, S., C. M. Cappellano, M. Ball, F. X. Francou, P. Normand, G. Helynck, *et al*. 2003. Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. *Appl. Environ. Microbiol.* **69:** 49–55.
7. Craig, J. W., F. Y. Chang, and S. F. Brady. 2009. Natural products from environmental DNA hosted in *Ralstonia metallidurans*. *ACS Chem. Biol.* **4:** 23–28.
8. Craig, J. W., F. Y. Chang, J. H. Kim, S. C. Obiajulu, and S. F. Brady. 2010. Expanding small-molecule functional metagenomics through parallel screening of broad-host-range cosmid environmental DNA libraries in diverse proteobacteria. *Appl. Environ. Microbiol.* **76:** 1633–1641.
9. Daniel, R. 2004. The soil metagenome – a rich resource for the discovery of novel natural products. *Curr. Opin. Biotechnol.* **15:** 199–204.
10. Daniel, R. 2005. The metagenomics of soil. *Nat. Rev. Microbiol.* **3:** 470–478.
11. Duan, C. J. and J. X. Feng. 2010. Mining metagenomes for novel cellulase genes. *Biotechnol. Lett.* **32:** 1765–1775.
12. Felnagle, E. A., E. E. Jackson, Y. A. Chan, A. M. Podevels, A. D. Berti, M. D. McMahon, and M. G. Thomas. 2008. Nonribosomal peptide synthetases involved in the production of medically relevant natural products. *Mol. Pharm.* **5:** 191–211.
13. Gabor, E. M., W. B. Alkema, and D. B. Janssen. 2004. Quantifying the accessibility of the metagenome by random expression cloning techniques. *Environ. Microbiol.* **6:** 879–886.
14. Gillespie, D. E., S. F. Brady, A. D. Bettermann, N. P. Cianciotto, M. R. Liles, M. R. Rondon, *et al*. 2002. Isolation of antibiotics turbomycin A and B from a metagenomic library of soil microbial DNA. *Appl. Environ. Microbiol.* **68:** 4301–4306.
15. Juncker, A. S., H. Willenbrock, G. Von Heijne, S. Brunak, H. Nielsen, and A. Krogh. 2003. Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci.* **12:** 1652–1662.
16. Kennedy, J., N. D. O'Leary, G. S. Kiran, J. P. Morrissey, F. O'Gara, J. Selvin, and A. D. Dobson. 2011. Functional metagenomic strategies for the discovery of novel enzymes and biosurfactants with biotechnological applications from marine ecosystems. *J. Appl. Microbiol.* **111:** 787–799.
17. Lim, H. K., E. J. Chung, J. C. Kim, G. J. Choi, K. S. Jang, Y. R. Chung, *et al*. 2005. Characterization of a forest soil

metagenome clone that confers indirubin and indigo production on *Escherichia coli*. *Appl. Environ. Microbiol.* **71:** 7768–7777.
18. MacNeil, I. A., C. L. Tiong, C. Minor, P. R. August, T. H. Grossman, K. A. Loiacono, *et al*. 2001. Expression and isolation of antimicrobial small molecules from soil DNA libraries. *J. Mol. Microbiol. Biotechnol.* **3:** 301–308.
19. McMahon, M. D., C. Guan, J. Handelsman, and M. G. Thomas. 2012. Metagenomic analysis of *Streptomyces lividans* reveals host-dependent functional expression. *Appl. Environ. Microbiol.* **78:** 3622–3629.
20. Nimchua, T., T. Thongaram, T. Uengwetwanit, S. Pongpattanakitshote, and L. Eurwilaichitr. 2012. Metagenomic analysis of novel lignocellulose-degrading enzymes from higher termite guts inhabiting microbes. *J. Microbiol. Biotechnol.* **22:** 462–469.
21. Nishie, M., J. Nagao, and K. Sonomoto. 2012. Antibacterial peptides "bacteriocins": An overview of their diverse characteristics and applications. *Biocontrol Sci.* **17:** 1–16.
22. Ono, A., R. Miyazaki, M. Sota, Y. Ohtsubo, Y. Nagata, and M. Tsuda. 2007. Isolation and characterization of naphthalene-catabolic genes and plasmids from oil-contaminated soil by using two cultivation-independent approaches. *Appl. Microbiol. Biotechnol*. **74:** 501–510.
23. Paik, H. D., S. S. Bae, S. H. Park, and J. G. Pan. 1997. Identification and partial characterization of tochicin, a bacteriocin offduced by *Bacillus thuringiensis* subsp *tochigiensis*. *J. Ind. Microbiol. Biotechnol.* **19:** 294–298.
24. Pushpam, P. L., T. Rajesh, and P. Gunasekaran. 2011. Identification and characterization of alkaline serine protease from goat skin surface metagenome. *AMB Express* **1:** 3.
25. Reis, J. A., A. T. Paula, S. N. Casarotti, and A. L. B. Penna. 2012. Lactic acid bacteria antimicrobial compounds: Characteristics and applications. *Food Eng. Rev.* **4:** 124–140.
26. Rondon, M. R., P. R. August, A. D. Bettermann, S. F. Brady, T. H. Grossman, M. R. Liles, *et al*. 2000. Cloning the soil metagenome: A strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl. Environ. Microbiol.* **66:** 2541–2547.
27. Torsvik, V., F. L. Daae, R. A. Sandaa, and L. Ovreas. 1998. Novel techniques for analysing microbial diversity in natural and perturbed environments. *J. Biotechnol.* **64:** 53–62.
28. Torsvik, V., J. Goksoyr, and F. L. Daae. 1990. High diversity in DNA of soil bacteria. *Appl. Environ. Microbiol.* **56:** 782–787.
29. Walsh, C. T. 2004. Polyketide and nonribosomal peptide antibiotics: Modularity and versatility. *Science* **303:** 1805–1810.
30. Wang, G. Y., E. Graziani, B. Waters, W. Pan, X. Li, J. McDermott, *et al*. 2000. Novel natural products from soil DNA libraries in a streptomycete host. *Org. Lett.* **2:** 2401–2404.
31. Wexler, M., P. L. Bond, D. J. Richardson, and A. W. Johnston. 2005. A wide host-range metagenomic library from a waste water treatment plant yields a novel alcohol/aldehyde dehydrogenase. *Environ. Microbiol.* **7:** 1917–1926.