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Metagenomic Analysis of *Streptomyces lividans* Reveals Host-Dependent Functional Expression

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Most functional metagenomic studies have been limited by the poor expression of many genes derived from metagenomic DNA in *Escherichia coli*, which has been the predominant surrogate host to date. To expand the range of expressed genes, we developed tools for construction and functional screening of metagenomic libraries in *Streptomyces lividans*. We expanded on previously published protocols by constructing a system that enables retrieval and characterization of the metagenomic DNA from biologically active clones. To test the functionality of these methods, we constructed and screened two metagenomic libraries in *S. lividans*. One was constructed with pooled DNA from 14 bacterial isolates cultured from Alaskan soil and the second with DNA directly extracted from the same soil. Functional screening of these libraries identified numerous clones with hemolytic activity, one clone that produces melanin by a previously unknown mechanism, and one that induces the overproduction of a secondary metabolite native to *S. lividans*. All bioactive clones were functional in *S. lividans* but not in *E. coli*, demonstrating the advantages of screening metagenomic libraries in more than one host.

The existing repertoire of microbially derived medically, agriculturally, and industrially useful enzymes and natural products originated from readily culturable organisms (28). However, the vast majority of microorganisms cannot be cultured with standard laboratory techniques, which necessitates alternative methods for accessing the metabolic potential of the uncultured organisms (33). Metagenomics, the sequence- and function-based analysis of the collective genomes of assemblages of organisms, provides such access (10, 15, 18, 36).

One approach to metagenomic analysis is functional metagenomics, which uses activity-based screens and selections to filter the genetic material from a community through a heterologous host. These screens reveal functional genes that code for desired biological activities, independent of their similarity to previously known genes. Functional metagenomics, therefore, can identify genes coding for biological activities of interest that would be missed with sequence-based analysis (17). Moreover, functional metagenomics couples a specific biological activity to genetic information, facilitating rapid identification and characterization of the genes and gene products involved in the biological activity. Among the diverse biological activities revealed using functional metagenomics are antimicrobial activity (8, 15, 24, 34), antibiotic resistance determinants (2), quorum-sensing mimics (16, 42), and enzymes with diverse functions (19, 20, 27). Many of these metabolites and activities would have been difficult to predict based on sequence information alone.

While the strength of functional metagenomics is the identification of biological activity in a manner independent of sequence data, the weakness of this approach is its dependence on the ability of the surrogate host to transcribe the metagenomic DNA efficiently, translate the mRNA to form a functional protein, produce the necessary cofactors and substrates, and localize the protein correctly. *Escherichia coli* is predicted to readily express 40% of environmental genes; however, this value drops to 7% for high-GC% actinomycete DNA (14). Therefore, functional metagenomics using *E. coli* as a heterologous host may underrepresent the metabolic potential of actinomycete species. The use of alternative hosts may increase the success of functional metagenomics by providing expression machinery suited to genes from diverse organisms (34). For example, *E. coli*, *Streptomyces lividans*, and *Pseudomonas putida* containing various antibiotic biosynthetic gene clusters produced variable levels of antibiotics (25), and six species of *Proteobacteria* differed in expression of metagenomic genes of interest (9). These studies demonstrated that functionally screening metagenomic libraries in alternative hosts provides access to metabolic potential different from that available in studies performed with *E. coli* alone.

The choice of a suitable host is a critical factor for maximizing biological activity detection in functional metagenomics (26). Factors to consider include simplicity in handling, favorable growth characteristics, availability of genetic tools, and appropriate cellular machinery for protein or metabolite production and activity (41). *S. lividans* makes an excellent host for functional metagenomics of soil bacterial communities, because *Streptomyces* species and other actinomycetes have abundant metabolic potential, are common soil inhabitants, and are evolutionarily distant from *E. coli*, thereby expanding the diversity of genes likely to be expressed in *E. coli* (14). The well-developed methods of genetic transfer from *E. coli* into *S. lividans* also contribute to the power of studies employing *Streptomyces* spp. as surrogate hosts. We chose *S. lividans* over other genetically tractable *Streptomyces* species such as *S. coelicolor* because *S. lividans* has a less active restriction-modification system, enabling it to accept foreign DNA more efficiently (39).

Two prior studies used *Streptomyces* as a heterologous host in sequence-based metagenomic analyses in which clones of interest

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were identified by sequence and then mobilized into a heterologous host for functional analysis (8, 38). Those studies demonstrated that *Streptomyces* expressed genes derived from metagenomic DNA, but the discovered genetic information was limited to genes amplified by PCR primers specific to several classes of secondary metabolites. Functional metagenomics has the potential to locate previously unknown metabolic capabilities using screens that identify active clones based on function rather than known signature sequences in the genes. The benefits of using *S. lividans* for identification of new biosynthetic potential are best exemplified in the first published use of a streptomycete heterologous host for functional metagenomics, employing high-performance liquid chromatography combined with electrospray ionization mass spectrometry (HPLC-ESIMS) in a screen to identify clones that produced members of the terragine and norcardamine natural product families (40). Importantly, that study reported a much higher frequency of biologically active clones than had been detected in *E. coli* or any other heterologous host (26). However, one weakness in that approach was the inability to quickly retrieve the metagenomic DNA for molecular analysis. Therefore, there was no simple technique available to demonstrate that the metagenomic DNA was causative of the observed biological activity, and subsequent genetic studies to target genes of interest were not performed.

Here we report the development and deployment of tools and methods for using *S. lividans* as a host for functional metagenomic libraries. We constructed libraries, conjugated them into *S. lividans*, screened them for functions in *S. lividans*, and characterized bioactive clones. The methodology described in this work provides the means to expand the accessible genes in functional metagenomic studies to include those of the metabolically rich actinomycete species.

MATERIALS AND METHODS

Construction of cosmid pMM436. Cosmid pOJ436 (5) was modified to contain PacI restriction sites that flanked *attP* and $int^{\phi C31}$. To accomplish this, each PacI site was added independently in a stepwise manner. For insertion of the first PacI restriction site, the NcoI-ClaI fragment of pOJ436 was subcloned into the corresponding sites of plasmid pANT841 (32), resulting in plasmid pANT841-NcoI/ClaI. QuikChange PCR-based mutagenesis (Agilent Technologies, Santa Clara, CA) was used to replace the HindIII site with a PacI site in plasmid pANT841-NcoI/ClaI by the use of primers PacI For (5′-GCCCGTTGCGCATGTTAATTAAGACCGATG GCCGGTTTG-3′) and PacI Rev (5′-CAAACCGGCCATCGGTCTTAAT TAACATGCGCAACGGGC-3′). Successful transformants were subsequently screened for PacI site insertion by restriction digestion. The NcoI-ClaI fragment containing the introduced PacI site was subcloned into the corresponding sites of pOJ436, resulting in plasmid pOJ436-PacI. A second PacI restriction site was inserted between the BamHI and SpeI sites of plasmid pOJ436-PacI by introducing a small DNA fragment containing a PacI restriction site flanked by BamHI and SpeI restriction sites by the use of primers PacI For (5′-GCCCGTTGCGCATGTTAATTAAGACCGATG GCCGGTTTG-3′) and PacI Rev (5′-CAAACCGGCCATCGGTCTTAAT TAACATGCGCAACGGGC-3′). Insertion of the PacI site was confirmed by sequencing, and the final cosmid was designated pMM436.

A second version of pMM436 containing a kanamycin resistance gene (*neo*) within the PacI-flanked fragment of pMM436 containing the *attP* site and $int^{\phi C31}$ gene was constructed to expedite the reconstruction of the cosmid once it was recovered from the *S. lividans* genome (see below). The construction of pMM436-Kan was accomplished by amplifying the *neo* gene from pSuperCOS1 (Stratagene) by the use of primers Neo-Up-SpeI (5′-CATACTAGTAAAGCAGGTAGCTTGCAGTGG-3′) and Neo-Down-SpeI (5′-CATACTAGTAACCTTTCATAGAAGGCGGC-3′). The amplicon was digested with SpeI and inserted into the SpeI restriction site of pMM436, generating plasmid pMM436-Kan.

Construction of *S. lividans* Δ*red* Δ*act* strain. The methods for deletion of *S. lividans* gene clusters were modified from previously reported protocols (25).

(i) *S. lividans* Δ*red* strain. Deletion of the prodiginine (*red*) biosynthesis gene cluster involved fusing the first 2 kb of the cluster to the final 2 kb, with concomitant removal of the intervening DNA. This was accomplished using the temperature-sensitive vector pKC1139 (5). Briefly, pKC1139 is a *Streptomyces-E. coli* shuttle vector with an origin of replication that is temperature sensitive in *Streptomyces*. The first 2 kb of the *red* cluster were cloned into the HindIII-BamHI restriction sites of pKC1139, while the final 2 kb of the *red* cluster were cloned into the BamHI-EcoRI restriction sites. The resulting plasmid, pKC1139Δred, was conjugated from *E. coli* into *S. lividans*, and the deletion of the intervening sequence was accomplished by successive temperature shifts and antibiotic selections and screens following standard protocols (37). Confirmation of the Δ*red* genotype was performed by PCR amplification. The primers used to construct pKC1139Δred were as follows: redD For (5′-GACGGCCAAG CTTCCTCGACCTTGTGGACCTCGTCGGTGCGCATCA-3′), redD Rev (5′-GATCATCGGGTCGTCTGGGATCCCGGTCGTCAGGCGCT GAGCAGGCTGGTGT-3′), redoxidase For (5′-CGCCTGACGACCGGG ATCCCAGACGACCCGATGATCCCCAACCAGTGG-3′), and redoxidase Rev (5′-CGGGAATTCGCGGGGTCAGTACACGTAGGGGACGA ACTTC-3′). The resulting strain was designated *S. lividans* Δ*red*.

(ii) *S. lividans* Δ*red* Δ*act* strain. Deletion of the actinorhodin (*act*) biosynthesis gene cluster followed the same protocol as outlined above. The pKC1139Δact plasmid was constructed using primers actVI For (5′-GAAAGCTTTCGGCAGCGCGTCAGGGTGTCA-3′) and actVI Rev (5′-GGGATCCCTACTGCCTGGTGCTCACCGTCCAC-3′) and primers actlysR2 For (5′-GGGATCCCACGAGGGTGGTTGGCGTCGGAACAA GGC-3′) and actlysR2 Rev (5′-CGGAATTCCAGGAAGCACAGGACGC CGAGGACGAAC-3′). The mutagenesis was performed using the *S. lividans* Δ*red* strain, resulting in *S. lividans* Δ*red* Δ*act*.

Construction of metagenomic libraries. pMM436 was prepared by digestion with HpaI followed by phosphatase treatment and digestion with BamHI and used in library construction.

(i) AKM1 library. Fourteen isolates from Alaskan soil were grown to saturation in 0.1× Trypticase soy broth. The cells were pelleted by centrifugation, resuspended in 23 ml of 10% (vol/vol) glycerol, and then frozen in 1.5-ml aliquots until use. Chromosomal DNA was extracted from 250 μl of cells by the use of an established protocol (30). DNA (0.75 μg) was partially digested to an approximate size of 30 to 40 kb with Sau3AI and ligated into the BamHI site of pMM436 (1.5 μg). The ligation mixture was packaged into phage by the use of MaxPlax Lambda packaging extracts (Epicentre, Madison, WI) and transduced into *E. coli* Epi300. Transductants were isolated by selection on LB containing apramycin (100 μg/ml). A total of 10,500 clones were isolated and then pooled by adding 600 μl of LB medium to each plate and scraping colonies into suspensions. All colonies were pooled, mixed, and resuspended in a final concentration of 10% (vol/vol) dimethyl sulfoxide (DMSO) and stored in 1.5-ml aliquots at −80°C. Restriction digestion analysis of 10 randomly chosen clones determined an average insert size of 35 kb.

(ii) AKM2 library. Cells were physically isolated from 100 g of Alaskan soil from Bonanza Creek Experimental Forest near Fairbanks, Alaska, by a previously published cell separation method (42). The cell pellets were flash frozen and stored at −80°C. Metagenomic DNA was extracted from cell pellets by the use of the protocol described above for AKM1. The optimal conditions for partial digestion of 0.2 μg of metagenomic DNA included digestion with 240 U of BclI at 50°C for 1.5 h, which resulted in fragments 30 to 40 kb in size. The digested DNA was dephosphorylated, ethanol precipitated, resuspended in 14 μl of water, and ligated with pMM436 (1.5 μg). Optimal ligation was achieved by incubating the ligating DNA at 18°C over 3 days in a ligation reaction mixture supplemented

TABLE 1 Primer sequences

Primer name[a]	Primer sequence (5′→3′)
Hydrox, Amido, Mel1,2 For	ACGGCCAGTGCCAAGCTTCCTAGAGGTCCTTGTCTGACGGAGC
Hydrox, Amido, Mel1,2 Rev	TGACATGATTACGAATTCGTTCCTCACTGGATGAAAGGCAGTC
MelC1,2 For	ACGGCCAGTGCCAAGCTTCGACTTCGACACGCTCGTCCGTACG
MelC1,2 Rev	TGACATGATTACGAATTCCGTGAGCGACACCACGGACATGTCC
Phospholipase For	ACGGCCAGTGCCAAGCTTGCATGGAGACCGTCGACAAGAAGG
Phospholipase Rev	TGACATGATTACGAATTCCCTCACCCTGTCCGATTGTCC
WhiG For	ACGGCCAGTGCCAAGCTTGCACTGCTGCTCGGACAGC
WhiG Rev	TGACATGATTACGAATTCCCAGCGTGAGCGCTGGTCAGC
S15 For	ACGGCCAGTGCCAAGCTTGCTTTGGCGAGACTCTCGATCTCG
S15 Rev	TGACATGATTACGAATTCCAGAGTTCTCAGGTTCGGCGATTACG
M28 For	ACGGCCAGTGCCAAGCTTGGAAGATTCGTTGCTTGCTGC
M28 Rev	TGACATGATTACGAATTCGGTAGCGGTCCTGGAAGATCAGC
Phospholipase del For	TCCCATGTCTACCGGCGGGTAACTGCCGCTGCTTTGATGGTGTAGGCTGGAGCTGCTTC
Phospholipase del Rev	TTACCGCCCCGACGCGATCACCGGGTAGTGGTCGGAGAGCATATGAATATCCTCCTTA
WhiG del For	ACACCCACGTTTCAGTTGAGAGGCTCAAGGTCACGTATGGTGTAGGCTGGAGCTGCTTC
WhiG del Rev	TCAGTCCTGGTCGGTGAGCTTGGCCCGCAGCTGAAGGACCATATGAATATCCTCCTTA
S15 del For	CGCCGGCATCATGCCGCCGACATCCGGGCAGATGTTATGGTGTAGGCTGGAGCTGCTTC
S15 del Rev	TCAGCGCATCACTGCAATCTCGAGGCGGGAGGGATGCGCCATATGAATATCCTCCTTA
M28 del For	CCGTACGGTCGAGTGCCGAGTGCGAGGCGGAGCGGGATGGTGTAGGCTGGAGCTGCTTC
M28 del Rev	TCAGGCGGGCGAGGTGAGAGCGCGGCGGGCTTGCGCCAACATATGAATATCCTCCTTA
MelC2 del For	TTCAACTGACCTTTCCAGCACCGGAGTTCATCTATCATGTGTGTAGGCTGGAGCTGCTTC
MelC2 del Rev	GAACGTGTAGTGGGCCGTGTGGTCCAGCAGATCCGCCGGCATATGAATATCCTCCTTA
Hydrox, Amido, Mel1,2 del For	CCTTGAGTAACTCTTGAGCGATTGTGGGATGTGCGCGTGTGTGTAGGCTGGAGCTGCTTC
Hydrox, Amido, Mel1,2 del Rev	CATACGAAACACCAACTGGCCCTTTTCCGGCGGCATATGCATATGAATATCCTCCTTA
Amido del For	GCCCATGGGCCGGGAGATGCGCGAGCGTGCTGCGTCATGTGTGTAGGCTGGAGCTGCTTC
Amido del Rev	TCAGTGGCCCGTGTCGCAGCAGCCCTCCCGCCGCATCAGCATATGAATATCCTCCTTA
Hydrox del For	CCTTGAGTAACTCTTGAGCGATTGTGGGATGTGCGCGTGTGTGTAGGCTGGAGCTGCTTC
Hydrox del Rev	TCAGCTCTTCGTGCCGTGCAGGGTCCGTGCCACCTTCGGCATATGAATATCCTCCTTA

[a] Hydrox, hydroxylase; Amido, amidohydrolase; del, deletion.

with 0.8 μl of 0.1 M ATP and 400 cohesive end units of ligase every 24 h. The ligated DNA was packaged into phage, transduced, selected, and stored (in pools of 4,000) as described above.

Conjugation of DNA libraries into *S. lividans* Δ*red* Δ*act*. Cosmid libraries were conjugated into *S. lividans* by standard triparental mating between *E. coli* Epi300 strains carrying cosmids, *E. coli* HB101 containing the helper plasmid pRK2013 (13), and *S. lividans* Δ*red* Δ*act* (21). Exconjugants were selected on MS agar (21) overlaid with apramycin (50 μg/ml) and naladixic acid (20 μg/ml).

Functional screening of metagenomic clones. Individual metagenomic clones in *S. lividans* Δ*red* Δ*act* were patched onto MS agar, incubated 30°C until sporulation was evident, and replica printed (31) onto three plates of ISP2 agar (35) and one plate of blood agar (Becton, Dickinson and Company, Sparks, MA). The original MS plate was retained as the master plate and stored at 4°C. The replication plates were incubated at 30°C for 5 days and screened for pigmentation, sporulation, and hemolytic activity. Tester organisms *Staphylococcus aureus*, *Pseudomonas aeruginosa* PAO1, and *E. coli* pJBA132 were inoculated into soft agar (Davis medium for *S. aureus* and *P aeruginosa* [12]) and LB (for *E. coli* [42]), poured over metagenomic clones on ISP2, and incubated at 37°C overnight. Plates were inspected visually for antibiotic activity and, in the case of *E. coli* pJBA132, microscopically with a green fluorescent protein (GFP) filter for fluorescence, which indicates induction of quorum sensing (Leica MZ FLIII; Leica Microsystems, Deerfield, IL) (excitation wavelength, 470/40 nm; barrier filter, 525/50 nm). The metagenomic library pools screened using *S. lividans* Δ*red* Δ*act* were also screened using *E. coli* Epi300 for pigments and alterations of colony morphology.

Confirming bioactivity of clones. Genomic DNA was extracted from active clones by the use of the DNA extraction protocol employed for library construction. The PacI restriction enzyme was used to excise the cosmid and metagenomic DNA from the *S. lividans* chromosome. The PacI enzyme was heat inactivated, and the DNA was self-ligated and transformed into *E. coli* Epi300. Transformants selected on LB plates with apramycin (100 μg/ml) contained metagenomic DNA and most of the vector. To confirm that the metagenomic DNA was responsible for the detected bioactivity, the PacI fragment of pMM436-kan containing *attP*, $int^{\Phi C31}$, and *neo* was inserted into PacI-digested cosmid. The reconstructed cosmid was then conjugated into *S. lividans* Δ*red* Δ*act*, and the resulting exconjugants and the clones in *E. coli* Epi300 were tested for the original bioactivity. Only those clones showing the original activity in *S. lividans* were characterized further.

Characterization of bioactive clones. Active clones with consistent phenotypes were each initially characterized using the same protocols, while further analysis was tailored to each clone. Several functional screens did not detect clones with bioactivities consistent enough to warrant characterization.

(i) Sequence analysis of bioactive clones. The cosmids of bioactive clones were partially digested and ligated into vector pSMART-HCKan by the use of a CloneSmart Blunt Cloning kit (Lucigen, Middleton, WI). The inserts were sequenced (UW Biotechnology Center) and then assembled into contigs by the use of SeqMan Lasergene software (DNASTAR, Madison, WI). Putative genes responsible for bioactivities were annotated using BLAST (Basic Local Alignment Tool) (3, 4) and SeqBuilder (DNASTAR). The sequences for these clones have been assigned GenBank accession numbers (see below).

(ii) Deletion analysis of bioactive clones. Insertional inactivation of targeted genes was performed using lambda Red mutagenesis in *E. coli* containing plasmid pKD46 (11). Lambda Red recombination was used to replace targeted genes with a chloramphenicol resistance gene amplified from the pKD3 template plasmid (11) by the use of unique primers for each insertion (Table 1). For clone 12, the insertional inactivation of both peptidase-encoding genes on the same cosmid was accomplished by inserting the chloramphenicol resistance gene into the gene coding for the S15-peptidase homolog, followed by FLP recombinase-based elimination

of the resistance gene to leave a "scar" (11). The subsequent inactivation of the second peptidase on the scar-containing cosmid was conducted using lambda Red-mediated mutagenesis of the other peptidase-encoding gene. The overall schemes for further characterization of clones are described below. After PCR confirmation of insertions and scar, the mutated cosmids were conjugated into *S. lividans* Δ*red* Δ*act* and tested for alteration of the original phenotype.

(iii) Cloning of candidate genes from bioactive clones. Candidate genes for observed bioactivity were inserted into pSET152, a vector that can be conjugated from *E. coli* and *S. lividans* (5). We used polymerase incomplete primer extension methods (22) with unique primers for each gene (Table 1) to amplify and insert target genes into pSET152. The plasmids were then conjugated into *S. lividans* Δ*red* Δ*act*, and the resulting strains were phenotypically characterized. *E. coli* Epi300 strains containing these plasmids were also screened for observed phenotypes.

(iv) Further characterization of bioactive clones. The biological activities of four clones were investigated further. Following lambda Red mutagenesis and cloning of the phospholipase-encoding gene from hemolytic clone AKM1-1, the constructs were screened in *S. lividans* Δ*red* Δ*act* for alteration of the hemolytic phenotype. The PCR primers used for detecting the potential presence of a phospholipase-encoding gene in clones 2 to 7 were Phos For (5′-TACCCCAACTGGGGCCAGGA-3′) and Phos Rev (5′-GTSGACTGGGCRTGGGTGCC-3′).

Lambda Red mutagenesis was used to inactivate the genes coding for MelC2, amidohydrolase, and hydroxylase homologs in pigment-producing clone 8. A second construct was created that replaced the MelC1-, MelC2-, amidohydrolase-, and hydroxylase-encoding genes with an antibiotic resistance gene. The constructs were screened for alterations of pigment production in *S. lividans* Δ*red* Δ*act*. Subsets of these four genes were also cloned into pSET152, with the first construct containing all four genes and the second containing only the *melC1* and *melC2* genes, resulting in plasmids HyAmMel1,2-pSET152 and Mel1,2-pSET152, respectively. The constructs were screened in *S. lividans* Δ*red* Δ*act* for pigment production. The amidohydrolase- and hydroxylase-encoding genes on HyAmMel1,2-pSET152 were inactivated using the forward primer for lambda Red deletion of the hydroxylase-encoding gene and the reverse primer for lambda Red mutagenesis of the amidohydrolase-encoding gene (Table 1), and the constructs were screened for alteration of pigment production in *S. lividans* Δ*red* Δ*act*.

The brown pigment was partially purified from clone 8 and the amidohydrolase mutant grown in YEME medium (21) at 30°C. Briefly, the cells were grown to saturation and pellets were formed by centrifugation at 2,400 × *g* for 10 min. The supernatant of each culture was passed through a 5-ml Dowex 50WX8 [H^+] ion exchange resin (Thermo Fisher Scientific, NJ) (100 to 200 mesh). The resin was washed with three volumes of water followed by elution using a step gradient of two column volumes each of 0.25, 0.5, 1.0, and 1.5 M ammonium hydroxide. Fractions were collected, flash frozen, and lyophilized. Once dried, the fractions were resuspended in 500 μl of water. The culture supernatant of *S. lividans* Δ*red* Δ*act* carrying pMM436 was treated in a similar manner as a negative control. The 1.0 M ammonium hydroxide elution fraction of each was further separated using reverse-phase high-performance liquid chromatography (HPLC). A 100-μl volume of solution containing partially purified brown pigment was injected into a C_{18} column (Vydac C18 small pore; Grace). The elution profile was as follows: 5 min using isocratic 100% buffer A (H_2O with 0.1% [vol/vol] trifluoroacetic acid)–0% buffer B (acetonitrile with 0.08% [vol/vol] trifluoroacetic acid); 30 min using a linear gradient from 100% buffer A—0% buffer B to 0% buffer A—100% buffer B; and a final isocratic step of 0% buffer A—100% buffer B. Elution of metabolites was monitored at 316 nm. Matrix-assisted laser desorption ionization–time of flight and electrospray ionization mass spectrometry analyses of these metabolites were inconclusive.

The genes coding for the peptidases in clone 12 were deleted individually and simultaneously using lambda Red mutagenesis, and the resulting constructs were screened for alteration of hemolytic activity in *S. lividans* Δ*red* Δ*act*. Each peptidase-encoding gene was also cloned into pSET152 and screened in *S. lividans* Δ*red* Δ*act* for hemolytic activity. Similarly, the gene in clone 10 coding for a homolog of WhiG was inactivated in the original clone and confirmed to be causative of the observed phenotype. The gene was also cloned into pSET152 for expression in *S. lividans* Δ*red* Δ*act*. All clones presented here were confirmed to be responsible for the phenotype.

16S rRNA gene sequencing and analysis. Genomic DNA from lysed cells was used as the template for PCR amplification with primers 27f (5′-AGRGTTTGATYMTGGCTCAG-3′) and 1492r (5′-GGYTACCTTG TTACGACTT-3′). Amplicons were sequenced by the UW Biotechnology Center (3, 4), and BLASTN was used to determine the species-level designation for each isolate.

Nucleotide sequence accession numbers. The clone sequences determined in the present study have been assigned GenBank accession numbers JQ430656, JQ430657, JQ430658, and JQ437404.

RESULTS AND DISCUSSION

Vector and host optimization. To optimize the functional screening of metagenomic DNA libraries, the proper vector and *S. lividans* strain background were needed. For vector optimization, we focused on generating a derivative of the *E. coli-Streptomyces* shuttle cosmid pOJ436 (5). This cosmid was targeted for modification because, although it stably maintains large DNA inserts in *E. coli* and *S. lividans*, it is not easily recoverable from the *S. lividans* genome once integrated into the *attB* site. To circumvent this issue, a PacI restriction site was introduced on each side of the $int^{\Phi C31}$ gene and *attP* site in pOJ436, resulting in cosmid pMM436. Once integrated into the *attB* site of the chromosome, these restriction sites were used to recover the majority of the cosmid along with the metagenomic DNA insert by digesting the chromosomal DNA with PacI, religating, and transforming the ligation mixture into *E. coli* for cosmid recovery by selection for apramycin resistance. PacI was used because its recognition sequence is not present in the *S. lividans* genome (http://www.xbase.ac.uk/genome/streptomyces-lividans-tk24).

We modified *S. lividans* to remove its native ability to produce two classes of pigmented antibiotics, actinorhodin and the prodiginines, by the use of the double-recombination protocol described previously by Martinez et al. (25). The modified strain was more amenable to detection of natural products encoded by heterologous DNA, because the mutant did not produce its own pigments or inhibit tester organisms. The resulting strain, *S. lividans* Δ*red* Δ*act*, did not produce actinorhodin or the prodiginines, as demonstrated by visual inspection of colonies growing on R2YE medium (data not shown).

Construction of AKM1 metagenomic library with DNA from cultured bacteria. As a model for libraries of greater complexity and for development of functional screens, we constructed a 10,500-member cosmid library (AKM1) in *E. coli* that carries 367.5 Mb of DNA derived from equal cell masses of each of the 14 Alaskan strains (Table 2). We conjugated the library from *E. coli* into *S. lividans* Δ*red* Δ*act*, and 7,200 clones (15% of the library) were stored as patches on MS medium.

Construction of AKM2 metagenomic DNA library from DNA extracted directly from Alaskan soil. A 71,000-clone library (AKM2), containing DNA extracted directly from soil, was generated in *E. coli*. With an average insert size of 35 kb, AKM2 contained 2,485 Mb of metagenomic DNA, the equivalent of ~500 average-size bacterial genomes. The library was conjugated into *S. lividans* Δ*red* Δ*act*, producing a collection of

TABLE 2 Identification of Alaskan soil bacterial isolates

Phylum	Species[a]
Bacteroidetes	*Chryseobacterium indoltheticum*
Actinobacteria	*Streptomyces lavendulae*
	S. spiroverticillatus
	S. somaliensis
	S. beijiangensis
	S. phaeochromogenes
	S. clavifer
	S. tauricus
	S. atratus
	S. microflavus
Betaproteobacteria	*Janthinobacterium lividum/J. agaricidamnosum*[b]

[a] Species designations are based on 16S rRNA gene sequences; species assignment indicates greater than 97% identity with type strain.
[b] Two sequences with the greatest identity to both species were isolated.

16,400 exconjugants, representing approximately 3% of the original library.

Functional screening in *S. lividans* Δ*red* Δ*act*. Four case studies of diverse metagenomic clones that demonstrated successful implementation of the described tools and methods designed to functionally screen metagenomic libraries in *S. lividans* and characterize the genes involved are provided below.

(i) Hemolytic clones from the culture-based AKM1 library. Seven hemolytic clones, designated clones 1 to 7 (Table 3), were identified in the AKM1 library. Clone 1 encoded a protein with 86% sequence identity to a putative phospholipase from *S. roseosporus* (ZP_04697445.1). This gene was targeted for further analysis, because members of the phospholipase family are known to have hemolytic activity (43). Hemolytic activity was abolished by inactivation of the gene encoding the phospholipase, and activity was recovered in a subclone containing the gene and 223 bp upstream of the start codon (Fig. 1A), indicating that the phospholipase is responsible for the hemolytic activity of clone 1. PCR analysis showed that each of the other hemolytic clones contained a phospholipase-encoding gene that was likely responsible for their hemolytic activity. None of the clones showed hemolytic activity in *E. coli*, which indicates that *S. lividans* was a better host for expression of these enzymes.

(ii) Pigment-producing clones from the culture-based AKM1 library. Two clones from the AKM1 library produced a

TABLE 3 Bioactive clones from metagenomic libraries

Clone	Library	Activity
1	AKM1	Hemolytic
2	AKM1	Hemolytic
3	AKM1	Hemolytic
4	AKM1	Hemolytic
5	AKM1	Hemolytic
6	AKM1	Hemolytic
7	AKM1	Hemolytic
8	AKM1	Brown pigment
9	AKM1	Brown pigment
10	AKM2	Spore pigment
11	AKM2	Spore pigment
12	AKM2	Hemolytic

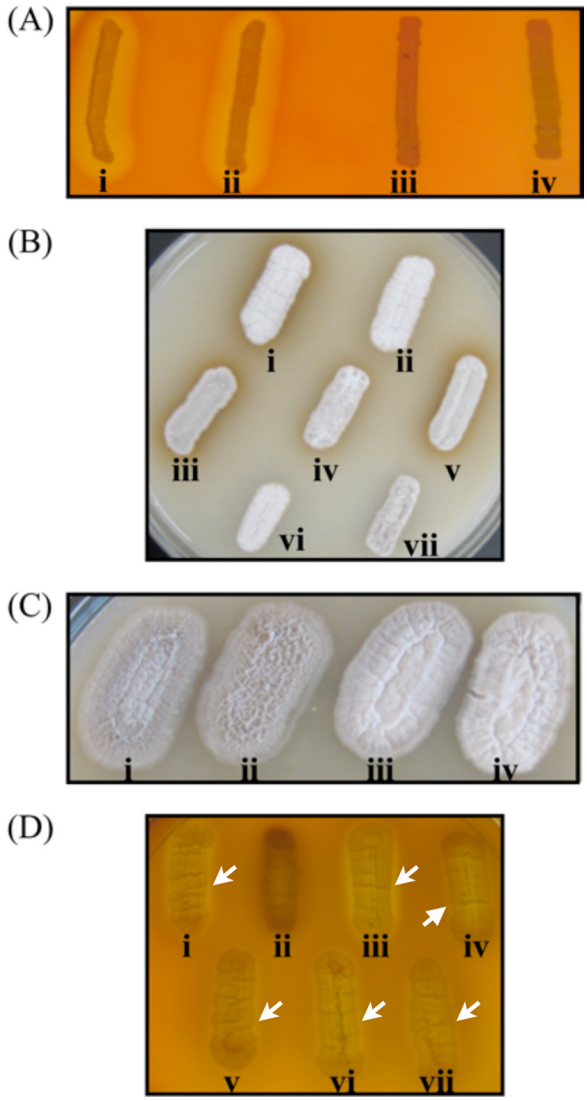

FIG 1 Functionally active metagenomic clones in *S. lividans*. (A) Hemolytic activity of *S. lividans* Δ*red* Δ*act* strains grown on blood agar plates. The photograph was taken from the bottom of the plate to more clearly show the lysis. Strains contained (i) original metagenomic DNA, (ii) pSET152 expressing putative phospholipase, (iii) original metagenomic clone with putative phospholipase inactivated, or (iv) empty cosmid vector. (B) Pigment production by *S. lividans* Δ*red* Δ*act* strains grown on MS agar. Strains contained (i) hydroxylase (Hy), amidohydrolase (Am), and MelC1,2-pSET152; (ii) HyAmMelC1,2-pSET152 with both Hy and Am inactivated; (iii) original metagenomic DNA; (iv) original metagenomic DNA with Am inactivated; (v) original metagenomic DNA with Hy inactivated; (vi) original metagenomic DNA lacking HyAmMel1,2; or (vii) empty cosmid vector. (C) Colony morphology and spore pigment production by *S. lividans* Δ*red* Δ*act* strains grown on MS agar. Strains contained (i) original metagenomic DNA, (ii) whiG:pSET152, (iii) original metagenomic DNA with *whiG* inactivated, or (iv) empty cosmid vector. (D) Hemolytic activity of *S. lividans* Δ*red* Δ*act* strains grown on blood agar plates. The photograph was taken from the bottom of the plate, and the white arrows note the lysis. Strains contained (i) original metagenomic clone, (ii) empty pSET152, (iii) S15:pSET152, (iv) M28:pSET152, (v) original metagenomic clone with S15 peptidase-encoding gene inactivated, (vi) original metagenomic clone with M28 peptidase-encoding gene inactivated, or (vii) original metagenomic clone with both putative peptidase-encoding genes inactivated.

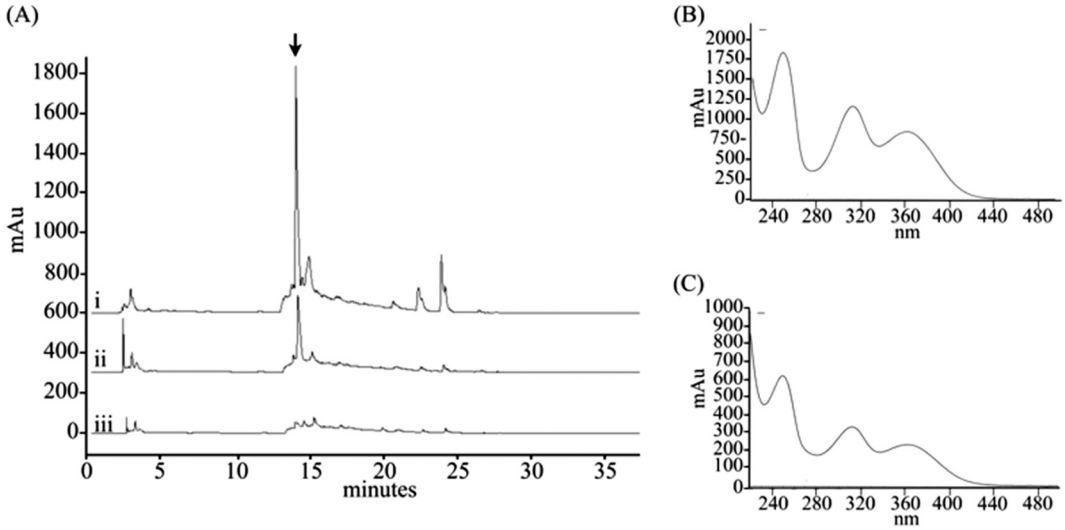

FIG 2 Brown pigment purification. (A) HPLC elution profile of metabolites purified from *S. lividans* Δ*red* Δ*act* strains containing (trace i) original metagenomic clone, (trace ii) original metagenomic clone with putative amidohydrolase gene inactivated, or (trace iii) empty cosmid vector. The arrow identifies the peaks in traces i and ii that were associated with a brown pigment. (B) UV-vis spectrum of the metabolite identified in trace i. (C) UV-vis spectrum of the metabolite identified in trace ii. mAu, milli-absorbance units.

brown diffusible pigment when grown on MS agar but did not produce the pigment in *E. coli*. Clone 8 (Table 3) contained genes coding for enzymes with 85% identity to MelC2 from *S. avermitilis* (NP_822312.1) and 72% identity to MelC1 from *S. viridochromogenes* (ZP_07301457.1), respectively. MelC1 and MelC2 are commonly associated with the production of the pigment melanin (7). MelC2 is a copper-dependent tyrosinase, and MelC1 is necessary for copper insertion into MelC2 and proper translocation of functional MelC2 through the twin-arginine translocation (TAT) system (7, 23). Mutational analysis confirmed that melC2 was critical for pigment production in the *S. lividans* Δ*red* Δ*act* host (Fig. 1B), and subcloning of *melC1* and *melC2* resulted in a low level of production of pigment.

To determine whether additional genes were needed for optimal pigment production, we inserted *melC1*, *melC2*, and two downstream genes coding for enzymes with 76% and 77% identity to a 2-polyprenyl-6-methoxyphenol hydroxylase from *S. avermitilis* (NP_822309.1) and a metallo-dependent amidohydrolase from *S. scabiei* (YP_003486560). The putative amidohydrolase protein has an N-terminal TolB domain, which may be important for interactions with the peptidoglycan (1), and a C-terminal metallo-dependent hydrolase domain. *S. lividans* Δ*red* Δ*act* containing this construct produced levels of brown pigment similar to those seen with the original clone (Fig. 1B). Inactivation of the gene coding for the putative hydroxylase had no impact on pigment production, but inactivation of the putative amidohydrolase-encoding gene resulted in a substantial reduction of pigment production (Fig. 1B). Simultaneous deletion of both genes reduced pigment production to a level comparable to that resulting from a deletion of just the amidohydrolase-encoding gene, thereby implicating only the amidohydrolase in pigment production.

There are two possible mechanisms by which the amidohydrolase might affect pigment production: (i) the enzyme might modify melanin during its biosynthesis or (ii) the enzyme might be involved in the formation of catalytically active tyrosinase (MelC2). In an attempt to discriminate between these two possibilities, we purified the pigment from both the original clone and the clone modified by deleting the putative amidohydrolase-encoding gene and analyzed the samples by HPLC and UV-visible light analysis (UV-vis). As expected, the strain lacking the amidohydrolase gene produced less pigment, but the pigments from the two strains were indistinguishable (Fig. 2), which suggests that the two molecules are structurally similar and that the putative amidohydrolase does not catalyze a modification of the pigment.

Another possibility is that the amidohydrolase is involved in processing the tyrosinase enzyme. The discovery of two forms of tyrosinase purified from the culture supernatant of *S. michiganensis* DSM 40015 provides support for this hypothesis, but further analysis is required to determine the role of the amidohydrolase protein in melanin biosynthesis (29). Importantly, melanin production was not observed from either the *E. coli* strain containing the original clone or the *E. coli* strain containing *melC1*, *melC2*, and the hydroxylase- and amidohydrolase-encoding genes on the pSET152 plasmid. At this time, we do not know whether the lack of activity in *E. coli* was due to expression, translation, posttranslational modification, or proper protein localization issues. Regardless, *S. lividans* was a superior host for identifying functionally active clones.

(iii) Alaskan soil AKM2 metagenomic library hemolytic clone. Screening the AKM2 library identified a clone with weak hemolytic activity (Fig. 1D). Sequence analysis revealed two genes whose encoded products have 39% and 58% identity to a member of the M28 peptidase family from *Pelagibacter bermudensis* (ZP_01441818.1) and the S15 peptidase family from *Acidovorax avenae* (ZP_06211296.1), respectively. To determine whether the peptidase-encoding genes were causative of the hemolytic activity, each gene was introduced into *S. lividans* Δ*red* Δ*act* and the strain was analyzed for hemolytic activity. Both genes conferred hemolytic activity on *S. lividans* Δ*red* Δ*act*, although the S15 peptidase homolog produced a larger zone of hemolysis than did the M28 homolog (Fig. 1D). Deletion of the S15 peptidase-encoding gene

from the original clone reduced hemolytic activity, and deletion of the M28 peptidase-encoding gene did not have any visible effect on the hemolytic activity. Any loss of hemolytic activity in this case may have been masked by the presence of the more active S15 peptidase.

To determine whether an additional gene(s) was required for hemolytic activity, we constructed a strain lacking the genes encoding both the S15 and M28 peptidases. This strain produced a zone of hemolysis similar to that seen with the strain mutated for the S15 peptidase but larger than that seen with the empty vector control. We conclude that we have found two genes encoding functional peptidases that are responsible for the hemolytic phenotype of clone 12. Further bioinformatic analysis revealed a gene between these two peptidases with 80% identity to a probable hydrolase-encoding gene from *Roseomonas cervicalis* (ZP_0689401.1), which may also contribute to the hemolytic activity. The 13-kb fragment of the metagenomic DNA encodes three peptidases as well as a dipeptide ABC transporter that may function in concert with the peptidases to scavenge amino acids from the environment. Further characterization of the final peptidase was beyond the scope of the current study. Note that the metagenomic DNA from this clone showed the highest level of identity (70% identity with 46% query coverage) with DNA from *Rhizobium leguminosarum* bv. *trifolii* WSM1325, an alphaproteobacterium. This clone did not show similar activity when tested in *E. coli* (a gammaproteobacterium), suggesting that *S. lividans* was more appropriate for discovering the biological activities tested in this study.

(iv) Alaskan soil metagenomic library AKM2 spore pigment production. Clones 10 and 11 had significantly darker spore pigmentation than the parent *S. lividans* Δ*red* Δ*act* strain that was not found when the metagenomic DNA was present in *E. coli*. Clone 10 also had significant colony wrinkling compared to the parent strain and was chosen for further characterization (Fig. 1C). Clone 10 contains a gene encoding a protein with 79% identity to sigma factor WhiG from *Kineococcus radiotolerans* (YP_001361163.1), a homologue of the sigma factor involved in regulating spore pigment production in *S. coelicolor* (6).

Deletion analysis and cloning of the gene encoding a putative sigma factor determined that it was necessary and sufficient for both increased spore pigment production and altered colony morphology in *S. lividans* Δ*red* Δ*act* (Fig. 1C). The putative metagenomic sigma factor has 73% identity (83% similarity) to the *S. coelicolor* protein WhiG, which regulates spore pigment expression. Based on this homology, one hypothesis is that the darker pigmentation is a result of the putative metagenomic sigma factor increasing expression of the genes associated with spore pigment production in *S. lividans*. Further analyses of how the presence of this gene influences colony morphology and pigmentation are needed to test this hypothesis.

Conclusion. Functional metagenomics in the heterologous *E. coli* host provides access to the metabolic potential of organisms that cannot be cultured in the laboratory; however, it suffers from poor expression of genes from metabolically diverse actinomycete species. Our goal was to develop tools and methods to expand the diversity of genes that could be expressed in our libraries by using *S. lividans* as the host for metagenomic libraries. To accomplish this, we developed (i) a cosmid vector that can be conjugated between *E. coli* and *S. lividans* and quickly recovered from both bacteria, (ii) an appropriate streptomycete host for the detection of natural products and pigments, (iii) efficient conditions for extracting and cloning metagenomic DNA from the soil, and (iv) phenotypic screens for detecting biologically active metagenomic DNA in *S. lividans*. Implementation of this system revealed 12 functionally active clones, containing a gene previously unknown to be involved in bacterial melanin biosynthesis, two different mechanisms responsible for hemolytic activity, and a putative sigma factor that may be responsible for elevating production of a native secondary metabolite biosynthetic cluster. All active clones were functional in the streptomycete host but not in *E. coli*. These results indicate that our tools and methods for constructing and screening metagenomic libraries in *S. lividans* enable discovery and characterization of bioactive clones that could not be found using *E. coli* as a host.

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